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U.S. Environmental Protection Agency
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Attn: 8(d) Health and Safety Data Reporting Rule (Notification/Reporting)

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Re: For Your Information Submission:
49 FR 46741 (November 28, 1984) [OPTS-84013; FRL-2725-1]
TSCA Section 8(d) Health and Safety Data Reporting

Dear Sir:

The enclosed information is submitted on behalf of Dow Corning Corporation, Midland, Michigan, 48686-0994, on a For-Your-Information (FYI) basis as a follow-up to submissions made concerning octamethylcyclotetrasiloxane (OMCTS), which chemical substance was the subject of a health and safety data rule issued under Section 8(d) of the Toxic Substances Control Act (TSCA) and with an effective date of December 28, 1984 (sunset date December 28, 1994), as codified at 40 CFR 716 (Health and Safety Data Reporting). The information presented in this submission was generated as part of our Siloxane Research Program. This program was the subject of a memorandum of understanding, dated April 9, 1996, between Dow Corning and EPA.

FYI-02-001418

Chemical Substance:

556-67-2 Octamethylcyclotetrasiloxane (OMCTS, D_4)

Title of Report:

IN VITRO METABOLISM OF OCTAMETHYLCYCLOTETRASILOXANE (D_4) BY HUMAN LIVER MICROSOMES

Dow Corning Corporation
2001-I0000-50850
November 15, 2001

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Dow Corning Corporation
Midland, Michigan 48686-0
Phone: (517) 496-4000
www.dowcorning.com

Manufacturer:

Dow Corning Corporation
2200 West Salzburg Road
Midland, Michigan 48686-0994

For purposes of this For-Your-Information (FYI) submission, the general INTERNAL designation on the attached health and safety report is waived by Dow Corning.

If you require further information regarding this submission, please contact Dr. Rhys G. Daniels, Senior Regulatory Compliance Specialist, Product Safety and Regulatory Compliance, at 989-496-4222 or at the address provided herein.

Sincerely,

Patrick W. Langvardt
Director of Health and Environmental Sciences
(989) 496-4626

RGD01384

DOW CORNING CORPORATION
HEALTH AND ENVIRONMENTAL SCIENCES
CONTRIBUTING SCIENTIST REPORT

Report No.	2001-I0000-50850
Title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Study No.	8956
External Study No.	XT052398
Test Article:	Octamethylcyclotetrasiloxane (D_4)
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Analysts:	Wesley Christian, B.S. Steve Loecker, M.Sc. Andrea R. Wolff, B.S. Greg Loewen, B.S. Beth Taylor, B.S.
Sponsor:	Dow Corning Corporation 2200 W. Salzburg Road Midland, MI 48686
Sponsor Monitor:	Kathleen P. Plotzke, Ph.D. Toxicology Manager
Testing Facility:	XenoTech, L.L.C. 3800 Cambridge Kansas City, KS 66103
Study Completion Date:	November 15, 2001
Security Statement:	Dow Corning Internal - This report may be reproduced and shared with any Dow Corning employee. The Director of HES must approve distribution outside the corporation. When this INTERNAL report is no longer needed, it may be placed in office wastebaskets for destruction.

FINAL REPORT 11/15/2001

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Total Number of Pages:	132

ABSTRACT

Octamethylcyclotetrasiloxane (D_4) is an industrial chemical and also used as an ingredient in selected personal care products to which humans are potentially exposed. This study was designed to determine the role of human liver microsomal enzymes in the *in vitro* metabolism of [^{14}C]-D_4. A binary gradient HPLC method with radio-detection was provided by the Sponsor to resolve and quantify D_4 and its major metabolites. After several preliminary experiments to establish experimental procedures, the reversed-phase HPLC method was validated based on the criteria described in this report. Following the HPLC method validation, [^{14}C]-D_4 was incubated with a pool of liver microsomes obtained from humans, saline-treated (control) rats or phenobarbital-treated rats to assess species differences and proportionality of metabolite formation with respect to incubation time and protein concentration (determination of the initial rate conditions). Additional experiments were conducted to identify the cytochrome P450 (CYP) enzymes involved in the metabolism of [^{14}C]-D_4 by human liver microsomes. A combination of the following two experimental approaches were taken: (1) metabolism of [^{14}C]-D_4 by individual recombinant human CYP enzymes; and (2) an evaluation of the effects of specific antibodies against selected CYP enzymes on the metabolism of [^{14}C]-D_4 by human liver microsomes.

Results of the study are summarized as follows:

1. [^{14}C]-D_4 was metabolized *in vitro* by human liver microsomes to a single metabolite (which was later named M8). In the range of substrate concentration examined (0.01 to 5 μM), M8 was detected when [^{14}C]-D_4 (at 1, 2, 3 and 5 μM) was incubated with a pool of human liver microsomes (1 mg protein/mL) for 60 min. The formation of M8 was proportional to substrate concentration.
2. When [^{14}C]-D_4 (3 μM) was incubated for 60 min with human liver microsomes ranging from 0.5 to 2 mg protein/mL, and up to 120 min at 1 mg protein/mL, formation of M8 was not proportional to protein concentration or incubation time. In addition, incubations of [^{14}C]-D_4 (5 μM and 10 μM) with human liver microsomes (1 mg protein/mL) for 30 and 60 min did not show proportionality between M8 formation and incubation time. In all cases (3, 5 and 10 μM), approximately 10% of [^{14}C]-D_4 was converted to M8. However, up to 34% of [^{14}C]-D_4 disappeared.
3. Proportionality of M8 formation with respect to protein concentration and incubation time was re-evaluated at a lower range of protein concentrations (0.01 to 1 mg protein/mL) and for a shorter incubation time (1 to 60 min). Again, the results showed that M8 formation was not proportional to protein concentration and incubation time.
4. [^{14}C]-D_4 (5 μM) was incubated with a pool of liver microsomes obtained from human, saline-treated (control) rats and phenobarbital-treated rats to assess species differences and

proportionality of metabolite formation with respect to incubation time and protein concentration (determination of the initial rate conditions). [^{14}C]-D_4 was converted *in vitro* by liver microsomes from phenobarbital-treated rats to at least eight metabolites. The metabolites were designated M1 through M8, based on their relative retention times, with M1 being the most polar and M8 being the least polar. M8 was the major metabolite formed. The M1 peak appeared to consist of multiple peaks that were not completely resolved by HPLC. M8 was also identified as the major metabolite observed in incubations with human liver microsomes and also in liver microsomes from saline-treated rats. Formation of several metabolites (but not M8) in the incubation of [^{14}C]-D_4 with liver microsomes from phenobarbital-treated rats (0.25 mg protein/mL for 0-20 min or 0-0.5 mg protein/mL for 10 min) was proportional to protein concentration and/or incubation time.

5. [^{14}C]-D_4 (3 µM) was incubated with recombinant human CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11. Microsomes from baculovirus-infected insect cells containing empty vector (which contains no human CYP activity) were included in the assay as a negative control. Two metabolites, namely M5 and M8, were formed by CYP2B6 and one metabolite (M8) was formed by CYP3A4. In the case of CYP2B6, the amount of M8 doubled when the concentration of CYP2B6 quadrupled. In all other cases, there was little or no relationship between M8 formation and either the concentration of CYP enzyme or incubation time. Similarly, M5 formation was not entirely proportional to incubation time and amount of CYP2B6.
6. Inhibitory polyclonal antibodies raised against rat CYP enzymes were incubated with a pool of human liver microsomes to examine their effects on the M8 formation from [^{14}C]-D_4. Polyclonal antibodies against CYP2B and CYP3A inhibited the conversion of [^{14}C]-D_4 to M8 by 32% and 34%, respectively.

In conclusion, [^{14}C]-D_4 was metabolized by human liver microsomes to one major metabolite (M8). Although the conversion of D_4 to M8 did not exceed 10%, the formation of M8 was not proportional to protein concentration or incubation time. The results of the experiment to assess [^{14}C]-D_4 binding to human liver microsomes suggest that the loss of radioactivity in the incubation sample was not due to the binding of [^{14}C]-D_4 and its metabolite(s) to the microsomal protein. Since D_4 is volatile, it is possible that one or more of its metabolites is volatile also, which would make it difficult to quantify. This is one possible explanation for the lack of proportionality of metabolite formation with incubation time and protein concentration. With human liver microsomes (and possibly liver microsomes from saline-treated rats), the results (lack of proportionality with incubation time and protein concentration) seem to suggest that only

a portion of the added D_4 is available to be metabolized, which might occur if D_4 bound to plastic, formed micelles, or formed a film on the surface of incubation medium. However, the observation that with microsomes from phenobarbital-treated rats caused extensive metabolism of D_4 would seem to argue against such an interpretation of assay artifact and may suggest that microsomal metabolism of D_4 in the uninduced system is a complex blend of enzyme action and inhibition. Based on the results of experiments with recombinant human CYP enzymes and polyclonal antibodies, it was concluded that [^{14}C]-D_4 is primarily metabolized *in vitro* to M8, and that CYP2B6 and CYP3A4 are largely responsible for its formation.

GLP COMPLIANCE STATEMENT

This Protocol was conducted in compliance with Good Laboratory Practices as described under the EPA Toxic Substances Control Act (TSCA) Federal Regulations 40 CFR Part 792, with the following exceptions:

Deviations from GLP regulations

1. A computer software program (Shimadzu Class-VP, version 4.2) was used to integrate HPLC peak areas. This HPLC computer system has not been validated at the Testing Facility.
2. There were several instances when initials and date on the worksheets were not entered on the day that the experiment was performed. Information was added at a later date and documented as "Late entry, GLP deviation". Since the missing information was available elsewhere or could be discerned by reviewing the data set, this deviation had no significant impact on the results of the study.
3. There were two instances when substrate mix or stop reagent were not marked as added (linearity experiment on 10/4/99 and cDNA experiment on 1/12/00). However, based on the result of HPLC analysis, it was clear that these solutions were added correctly. Thus, this deviation did not negatively impact on the results of the study.

Protocol deviations

1. The protocol states that the final concentration of β-NADPH was to be 50-250 μM (target concentration). There were five instances when the final concentration exceeded 250 μM (determined spectrophotometrically). The highest concentration measured was 300 μM. Since the target concentration was designed to provide an excess amount of β-NADPH in the incubation mixture, having 300 μM (20% higher than the upper end of the target concentration) will not have any negative impact on the results of the study.
2. Protocol Amendment-6 was issued to document an experiment to assess the effect of incubation time and protein concentration on the metabolism of D_4 by liver microsomes from phenobarbital-treated rats (Time and Protein-1, PB-rat). The protein concentration stated in the amendment was 0.25, 0.5 and 1.0 mg protein/mL. After the conduct of the experiment and data review, which suggested excessive metabolism of D_4, this experiment was repeated as "Time and Protein-2 (PB-rat)" at a protein concentration of 0.125, 0.25 and 0.5 mg protein/mL. Since there was no amendment that documented this change, this was a protocol deviation. However, this change had no negative impact on the outcome of the study.

Etsuko Usuki 11/15/01
Etsuko Usuki, Ph.D. Date
Study Director

Kathy P. Plotzke 11-13-01
Kathy Plotzke, Ph.D. Date
Sponsor Monitor

QUALITY ASSURANCE STATEMENT

The following table summarizes dates of Quality Assurance Unit inspections and reporting to the Study Director and Management for Protocol XT (052398).

Quality Assurance Unit Inspections

Procedure/XenoTech SOP Followed	Date of			
	QAU Inspection	QAU Report	Report to Study Director	Report to Management
Experimental Phase/QAU05.04				
HPLC method validation/L0080.01	11/16/99	11/16/99	11/16/99	11/29/99
Effect of time, protein and substrate concentration/NA	11/18/99	11/19/99	11/19/99	11/29/99
Data audit/QAU06.05				
Preliminary Evaluation #1/NA	09/10/99	09/13/99	09/13/99	09/13/99
Preliminary Evaluation (Dry run)/NA	10/03/99	10/08/99	10/08/99	10/18/99
Preliminary Evaluation #2/NA	10/03-04/99	10/08/99	10/08/99	10/18/99
Preliminary Evaluation #3/NA	10/04-05/99	10/08/99	10/08/99	10/18/99
Preliminary Incubations/NA	11/17/99	11/22/99	11/22/99	11/29/99
Linearity Experiment/NA	11/17/99	11/22/99	11/22/99	11/29/99
Method Validation #1/NA	11/22/99	11/22/99	11/22/99	11/29/99
Method Validation #2/NA	11/17/99	11/22/99	11/22/99	11/29/99
Method Validation #2 Repeat/NA	11/17-18/99	11/22/99	11/22/99	11/29/99
Method Validation #3/NA	11/18/99	11/22/99	11/22/99	11/29/99
Method Validation #3 Repeat/NA	*11/17-18, 22/99	11/22/99	11/22/99	11/29/99
Effect of time, protein and substrate concentration/NA	12/05/99	12/06/99	12/06/99	12/07/99
Protein Suitability/NA	02/03/00	02/04/00	02/04/00	02/11/00
Effect of time, protein and substrate concentration #2/NA	02/03/00	02/04/00	02/04/00	02/11/00
cDNA experiment/NA	02/21/00	02/25/00	02/25/00	02/29/00
Covalent binding experiment/NA	03/08/00	03/13/00	03/13/00	03/20/00
Time and protein (Phenobarbital induced rat)/NA	04/17/00	04/24/00	04/24/00	05/08/00
Time and protein #2 (Phenobarbital induced rat)/NA	05/26/00	05/26/00	05/26/00	06/26/00
Rat microsome comparison experiment/NA	06/14-15/00	06/16/00	06/16/00	06/26/00
Species comparison experiment/NA	06/15/00	06/16/00	06/16/00	06/26/00
Species comparison experiment #2/NA	07/25/00	07/31/00	07/31/00	09/22/00
β-RAM Troubleshooting/NA	08/20/00	08/21/00	08/21/00	09/08/00
cDNA experiment #2/L7060.01	08/20/00	08/21/00	08/21/00	09/08/00
Antibody experiment/NA	09/12/00	09/15/00	09/15/00	09/15/00
Draft report audit/Q0070.01				
Draft report	07/26-27, 30-31, 2001	07/31/01	08/01/01	08/09/01
Draft report follow-up	08/09/01	08/09/01	08/09/01	08/27/01

*The date that the assay was individually audited was not recorded; however, the assay was audited with in the time frame specified.

This report has been reviewed by the Quality Assurance Unit in accordance with the Environmental Protection Agency (EPA) Good Laboratory Practice (GLP) Regulations, 40 CFR Part 792.

The data were audited for compliance with the Good Laboratory Practice regulations. The audit findings have been reported to Management and the Study Director. The results of this report accurately reflect the raw data for the study.

Mary M. Lewis — Quality Assurance Unit Representative

1 Nov 01 — Date

STUDY INFORMATION

Study Initiation Date:	6/29/99
Experimental Start Date:	9/02/99
Experimental Termination Date:	9/07/00
Study Completion Date:	11/15/01
Protocol No.	XT052398
Protocol Title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech, LLC 3800 Cambridge Kansas City, KS 66103 Tel. (913) 588 7530 Fax. (913) 588 7572
Sponsor:	Dow Corning Corporation 2200 West Salzburg Road Midland, Michigan 48686-0994
Participating Personnel:	
Study Director:	Etsuko Usuki, Ph.D., Senior Scientist XenoTech, LLC
Study Manager:	Ajay Madan, Ph.D., Chief Scientific Officer XenoTech, LLC
Report reviewer:	Andrew Parkinson, Ph.D., CEO XenoTech, LLC
Quality Assurance:	Kelly Bunce, B.S., QAU Manager, XenoTech, LLC
Analysts:	Wesley Christian, B.S. Scientist, XenoTech, LLC Andrea Wolff, B.S. Scientist, XenoTech, LLC Greg Loewen, B.S. Scientist, XenoTech, LLC Steve Loecker, M.S. Scientist, XenoTech, LLC Beth Taylor, B.S. Scientist, XenoTech, LLC
Sponsor Monitor and Sponsor's Representative:	Kathy Plotzke, Ph.D. Tel. (517) 496 8046 Fax (517) 496 5595
Protocol Summary:	This protocol was designed to determine the role of human liver microsomal enzymes in the *in vitro* metabolism of D_4. Part 1 of this protocol was designed to develop and

validate an HPLC procedure to resolve and quantify D_4 and its major metabolites. Part 1 of this protocol was also designed to determine if D_4 was metabolized by liver microsomes from humans or rats. Part 2 of this protocol was designed to characterize the metabolism of D_4 by human CYP enzymes and to identify which specific CYP enzyme or enzymes are responsible for metabolizing D_4.

Records:

All raw data, processed data (paper and electronic), study specific correspondence documentation, records, protocols (including amendments) and final reports generated from this study are archived by the Testing Facility.

Regulatory Compliance:

This study was entered onto the Testing Facility's master schedule of regulated studies. The study was conducted in accordance with the Environmental Protection Agency (EPA) Good Laboratory Practice (GLP) Regulations, 40 CFR Part 792 as described by the EPA Toxic Substances Control Act (TSCA).

Timetable of Study Conduct:

The following table summarizes the analysis dates, the participating analysts and the attending personnel from the Quality Assurance Unit for critical phases of protocol XT052398. This is not a Quality Assurance Report (see page 6).

Procedure	Date	Responsible Personnel	QAU
Protocol finalized	6/29/99	EU, AM, KP	KB
Test article received	7/09/99	EU	
Preliminary evaluation 1	9/2/99-9/3/99	WC	
Dry run (practice run without D_4)	9/13/99	WC, AW	
Preliminary evaluation 2	9/15/99-9/16/99	WC, AW	
Preliminary evaluation 3	9/20/99-9/21/99	WC, AW	
Preliminary incubations	9/23/99-9/29/99	WC, AW	
HPLC method validation			
Linearity experiment	10/04/99-10/06/99	WC, AW	
Method validation 1	10/11/99-10/15/99	WC, AW	
Method validation 2	10/26/99	WC, AW	
Method validation 2 (repeat)	11/01/99-11/03/99	WC, AW	
Method validation 3	11/08/99-11/10/99	WC	
Method validation 3 (repeat)	11/16/99-11/17/99	WC, AW	KB
Time and Protein 1 (HLM)	11/18/99-11/22/99	WC, AW	KB
Protein suitability	11/29/99	WC	
Time and Protein 2 (HLM)	11/3099-12/03/99	WC, AW	
Recombinant CYP enzymes	1/12/00-1/18/00	WC, AW	
Assessment of binding of D_4 to HLM	2/24/00	WC, AW	
Time and protein 1 (PB rat)	3/20/00-4/10/00	WC, AW	
Time and protein 2 (PB rat-repeat)	4/10/00-4/14/00	WC, AW, GL	
Rat microsome comparison	4/25/00-5/1/00	WC, GL	
Species comparison	6/05/00	WC, GL	
Species comparison 2 (repeat)	6/22/00-6/26/00	WC, GL	
Time and protein with recombinant CYPs	7/26/00-8/01/00	WC, BT	
Antibody inhibition	8/09/00-8/11/00	WC	
Sample preparation for HPLC analysis by the Sponsor (Amendment 9: Appendix 7)	9/07/00	GL	
Draft report	7/19/01	EU, AM, KP	KDM
Final report	11/01/01	EU	

AM, Ajay Madan; AW, Andrea R. Wolff; BT, Beth Taylor; EU, Etsuko Usuki; KDM, Kimberly D. Manthei; KP, Kathy Plotzke; KB, Kelly Bunce; WC, Wesley Christian; GL, Greg Loewen; HLM, Human liver microsomes and PB rat, Phenobarbital-treated rat.

SIGNATURE PAGE

The undersigned have read and approved this report:

11/15/01

Ajay Madan, Ph.D. **Date**
Chief Scientific Officer, Study Manager
XenoTech, L.L.C.
3800 Cambridge Tel. (913) 588 7509
Kansas City, KS 66103 Fax. (913) 588 7572

11/15/01

Etsuko Usuki, Ph.D. **Date**
Senior Scientist, Study Director
XenoTech, L.L.C.
3800 Cambridge Tel. (913) 588 7495
Kansas City, KS 66103 Fax. (913) 588 7572

11-13-01

Kathy Plotzke, Ph.D. **Date**
Sponsor Monitor
Dow Corning Corporation
2200 West Salzburg Road Tel. (517) 496 8046
Midland, MI 48686-0994 Fax. (517) 496 5595

INTRODUCTION AND STATEMENT OF OBJECTIVE

Octamethylcyclotetrasiloxane (D_4, **Figure 1**) is a clear, odorless, synthetically derived silicone fluid. It is a highly volatile fluid with a vapor pressure of 1 mm at 25 °C[1] and a water solubility of ~50 ppb.[2] D_4 is used principally as an intermediate in the industrial manufacture of polydimethylsiloxane, a silicone polymer that is used widely in industrial and consumer applications. Secondarily, D_4 is used as an ingredient in selected household and personal care products. Because of these widespread uses and the potential for human exposure, studies have been initiated to examine the biological fate and effects of this material.

Previous studies have shown that inhalation exposure of Fischer 344 rats to D_4 causes a phenobarbital-type induction of several liver microsomal drug-metabolizing enzymes, including CYP2B.[3,4] In addition, a study at the Testing Facility has shown that: (1) D_4 is a noncompetitive inhibitor of rat CYP2B1/2 activity with an estimated Ki value of 0.11 μM, (2) D_4 is a noncompetitive inhibitor of human CYP2B6 activity with an estimated Ki value of 3.6 μM, (3) D_4 is a competitive inhibitor of human CYP1A2 activity with an estimated Ki value of 12 μM, and (4) D_4 is a noncompetitive inhibitor of human CYP2D6 and CYP3A4/5 activities with estimated Ki values of 14 and 11 μM, respectively.[5]

This protocol was designed to determine the role of human liver microsomal enzymes in the *in vitro* metabolism of D_4. Part 1 of this protocol was designed to develop and validate an HPLC procedure to resolve and quantify D_4 and its major metabolites. Part 1 of this protocol was also designed to determine if D_4 was metabolized by liver microsomes from humans or rats. Part 2 of this protocol was designed to characterize the metabolism of D_4 by human CYP enzymes and to identify which specific CYP enzyme or enzymes are responsible for metabolizing D_4.

EXPERIMENTAL PROCEDURES

Materials

Chemicals

ß-NADPH, sucrose, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). EDTA, anhydrous ethanol and tetrahydrofuran (THF) were purchased from Aldrich (Milwaukee, WI). Potassium phosphate was purchased from J.T. Baker (Phillipsburg, NJ). Molecular sieves were purchased from Fischer Scientific (Pittsburgh, PA). All solvents were of HPLC grade. Ultima Flo M™ scintillation cocktail was purchased from Packard Instrument Co. (Meriden, CT). Water obtained from the Millipore® water purification system was used to prepare aqueous solutions.

Antibodies

Rabbit polyclonal antibodies against rat CYP2B1/2 or rat CYP3A1/2 and rabbit preimmune IgG were prepared at the Testing Facility and used for this study.

Test article

[^{14}C]-D_4 (D_4 which was randomly labeled with ^{14}C) was received from Wizard Laboratories, Inc. (West Sacramento, CA) on July 9, 1999 together with a Certificate of Analysis (**Appendix 1**). In addition, a characterization report (report No. 1999-I0000-47184) on the test article was provided by the Sponsor (**Appendix 2**).

Quantity received:	1.0 mCi
Specific activity:	20.62 Ci/mol
Molecular weight:	296.62 g/mol
Lot number:	990316
Solvent used for dissolution:	Anhydrous ethanol
Concentration of stock solution:	9.7 mM
Clarity in anhydrous ethanol:	Clear
Purity:	99.67%
Storage of stock solution:	Anhydrous ethanolic solution was stored in crimp capped clear plastic vials at -80±5°C.

Handling of [^{14}C]-D_4 and determination of concentration

Upon receipt, [^{14}C]-D_4 (in a glass ampoule) was dissolved in 5 mL anhydrous ethanol (500-µL rinse for 10 times) on ice bath (dry ice in acetone, to avoid evaporation of D_4) and transferred to a plastic crimp-top vial (Weaton 15-mL polypropylene serum vial). Anhydrous ethanol (stored with molecular sieves to absorb dissolved moisture) was drawn with a plastic syringe (B-D, 1 mL syringe with a 23-gauge stainless steel needle) fitted with a stainless steel adaptor to filter particulate matter through a glass filter disc (Whatman, polypropylene membrane filter, 0.2 µm). The 5-mL stock solution was aliquotted (500 µL) in ten 1-mL polypropylene crimp-top vials. This

solution served as a stock of [^{14}C]-D_4 in anhydrous ethanol (9.7 mM; the concentration was determined by measuring the amount of radioactivity in the aliquots by liquid scintillation counting). This solution was stored at -80±5°C, and was used as needed for the preparation of the substocks (working solutions) in ethanol. The final concentration of ethanol in the incubation was 0.5(v/v)%. On the day of each experiment, the substocks were prepared and mixed with buffer mix (described later) and liver microsomes to prepare substrate mix. The concentration of [^{14}C]-D_4 present in the substrate mix was determined by measuring the amount of radioactivity by liquid scintillation counting with a Packard 2900TR Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL).

Test system

Human liver microsomes (pool of 15 individuals) were used for this study. These human liver microsomes were prepared at the Testing Facility according to SOP XT03 and characterized with respect to the activities of various P450 enzymes (**Appendix 3**). In addition, liver microsomes from phenobarbital (PB)-treated (CYP2B-induced) and saline-treated (control) male Sprague Dawley rats were used for this study. These were also prepared and characterized at the Testing Facility (**Appendix 3**).

HPLC Method development, preliminary evaluations and method validation

A binary gradient reversed-phase HPLC method was transferred from the Sponsor to the Testing Facility. Several preliminary evaluations were conducted with incubations of [^{14}C]-D_4 with human liver microsomes and liver microsomes from PB-treated rats to detect metabolite(s), as described below. The HPLC method was then validated at the Testing Facility (**Appendix 4**).

HPLC method

[^{14}C]-D_4 and its metabolites were resolved and quantified by a binary gradient reversed-phase HPLC method, which used a Shimadzu LC-10A HPLC system equipped with a SIL-10A autosampler (Shimadzu Scientific Instruments, Columbia, MD) and an IN/US β-RAM model 2-radio detector (IN/US, Tampa, FL). The analytical column was an Alltima C-18, (Part No. 88056, Column No. 99050599, Lot No. 1062, 4.6 mm x 250 mm, 5 µm particle, Alltech, Deerfield, IL) preceded by an Alltima C-18 guard column (Part No. 96080, 5 µm particle). HPLC analysis was performed at ambient temperature. [^{14}C]-D_4 and metabolites were quantified from a calibration curve constructed with zero-time incubations containing different concentrations of [^{14}C]-D_4. A computer software program (Shimadzu Class-VP, version 4.2) was used to integrate peak areas. Mobile phase A was Millipore® water, whereas mobile phase B was 100% acetonitrile. The total flow rate was 1.0 mL/min and typical injection volume was 200 µL. The ratio of HPLC effluent to Ultima-Flo M scintillation cocktail was 1:3. The β-RAM flow cell volume was 500 µL. The gradient profile is shown below.

Gradient profile

Time (min)	% of mobile phase B	Gradient profile
0-20.0	0	Hold
20.0-40.0	0 to 100	Linear gradient
40.0-50.0	100	Hold
50.0-55.0	100 to 0	Linear gradient
55.0-70.0	0	Re-equilibrate

Preliminary evaluations (1, 2, 3 and "dry run")

During several preliminary evaluations, [^{14}C]-D_4 (5 and 332 µM) was incubated with liver microsomes from human (pool of 15, 0.5-1 mg protein/mL) or PB-treated rats (pool of 21 PB-0.5 mg protein/mL) in a buffer containing potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM, pH 7.4), and β-NADPH (50-300 µM) at the final concentrations indicated. The substrate mixture was prepared by mixing the buffer solution described above, [^{14}C]-D_4 (in anhydrous ethanol) and human liver microsomes. In addition, a "dry run" was performed, which was a practice session where [^{14}C]-D_4 was replaced with anhydrous ethanol. For actual incubations of [^{14}C]-D_4, the presence of microsomes was necessary to solubilize [^{14}C]-D_4 in buffer. Incubations were carried out at 37±1°C in 1-mL crimp-capped plastic vials for zero, 40 and 60 min. The final incubation volume was either 800 or 900 µL. Reactions were initiated by adding β-NADPH with a plastic syringe (B-D, 1 mL syringe with a 23-gauge stainless steel needle) and were terminated by transferring one-half of the incubation volume (400 or 450 µL) of the final incubation volume to a crimp-capped glass HPLC vial containing an equal volume of THF (stop reagent). All the sample transfer was performed by a plastic syringe. Tubes were centrifuged at 920 x *g* for 10 min at 10°C to remove precipitated protein. Following centrifugation, aliquots (200 µL) of the supernatant fractions were analyzed by HPLC (radiometric detection). No-substrate, no-NADPH, no-protein, and zero-time samples served as blanks.

Preliminary incubations

[^{14}C]-D_4 (0.01-5 µM, 0.17 nCi-82.5 nCi/incubation) was incubated with human liver microsomes (1 mg protein/mL) in a buffer containing potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM, pH 7.4), and β-NADPH (220 µM) at the final concentrations indicated (final incubation volume 800 µL). Incubations were carried out at 37±1°C in 1-mL crimp-capped plastic vials for zero and 60 min. Reactions were initiated by adding β-NADPH with a plastic syringe (B-D, 1 mL syringe with a 23-gauge stainless steel needle) and were terminated by transferring a half volume (400 µL) of the final incubation volume to a crimp-capped glass HPLC vial containing an equal volume of THF (stop reagent). Tubes were centrifuged at 920 x *g* for

10 min at 10°C to precipitate protein. Following centrifugation, aliquots (200 µL) of the supernatant fractions were analyzed by HPLC (radiometric detection). No-substrate, no-NADPH, no-protein, and zero-time samples served as blanks.

Based on the results of these preliminary evaluations, the final incubation procedure was established as follows. For zero-time incubations, no transfer of the substrate mix to incubation vials occurred. Instead, 350 µL of the substrate mix was transferred with a plastic syringe to a crimped vial containing 400 µL of THF to which 50 µL of β-NADPH was then added. For the actual incubation samples, 700 µL of substrate mix was added to a plastic vial and then crimped. To start the incubation, 100 µL of β-NADPH was added, by syringe, and the vial was then placed in the incubator. At the completion of the incubation (*e.g.*, after 60 min), a 400 µL-aliquot of the incubation sample was withdrawn by syringe and then transferred to a crimped vial containing 400 µL of THF to stop the reaction.

Time and protein-1 (Human liver microsomes)

The effect of incubation time and protein concentration on the rate of metabolite formation for [^{14}C]-D_4 was determined. [^{14}C]-D_4 (3 µM, 49.5 nCi/incubation) was incubated with a single concentration of pooled human liver microsomes (1 mg protein/mL) for multiple time periods (0, 15, 30, 45, 60 and 120 min) in 800-µL incubation mixtures containing potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM, pH 7.4) and β-NADPH (240 µM) at the final concentrations indicated. Additionally, [^{14}C]-D_4 (3 µM, 49.5 nCi/incubation) was incubated with three concentrations of human liver microsomes (0.5, 1.0 and 2.0 mg protein/mL) for a fixed period of 60 min. In addition, [^{14}C]-D_4 (5 µM and 10 µM; 82.5 and 165.0 nCi/incubation, respectively) was incubated with a single concentration of human liver microsomes (1 mg protein/mL) for multiple time periods (0, 30 and 60 min). No-substrate, no-NADPH and no-protein samples served as blanks. The remaining procedure was essentially the same as that described above (Preliminary Incubations).

Protein suitability and Time and protein-2 (Human liver microsomes)

The effect of incubation time and protein concentration on the rate of metabolite formation for [^{14}C]-D_4 was reassessed with lower protein concentrations and shorter incubation times. The presence of microsomes was required to solubilize [^{14}C]-D_4 in buffer. Therefore, prior to this reassessment, the minimum amount of protein needed to retain [^{14}C]-D_4 in the buffer solution was determined (protein suitability experiment). [^{14}C]-D_4 (0.4, 1.6 and 4 µM) solution was prepared in buffer mix in the presence of human liver microsomes (1 mg protein/mL and 0.05 mg protein/mL) and the percent recovery of [^{14}C]-D_4 in substrate mix was determined by measuring concentrations of [^{14}C]-D_4 present in the substrate mix by scintillation counting and comparing the observed and theoretical concentrations. Once the minimum protein concentration needed in the incubation mixture was determined, [^{14}C]-D_4 (3 µM, 49.5 nCi/incubation) was incubated with a single concentration of human liver microsomes

(0.1 mg protein/mL) for 0, 1, 5, 10, 20, 30 and 60 min. In addition, [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was incubated with human liver microsomes (0.01, 0.05, 0.1, 0.2, 0.5 and 1.0 mg protein/mL) for a fixed time (15 min). No-substrate, no-NADPH and no-protein samples served as blanks. The remaining procedure was essentially the same as that described in the section of Preliminary Incubations.

Assessment of binding of D_4 to human liver microsomes

This experiment was designed to assess the possibility of [^{14}C]-D_4 binding to protein and causing the loss of radioactivity that could not be accounted for through the formation of metabolite in the past experiments. [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was incubated with a single concentration of human liver microsomes (0.1 mg protein/mL) for 0, 20, 40 and 60 min. Radioactivity in 100-μL aliquots were determined by liquid scintillation counting in the substrate mix, incubation samples after the addition of β-NADPH, quenched incubation samples (containing THF), supernatant fractions of the quenched incubation sample (after precipitated protein was removed by centrifugation) and suspension of the microsomal pellet (after protein was pelleted by centrifugation, 800 μL of anhydrous ethanol was added to the pellet to make a suspension).

Time and protein (Liver microsomes from PB-treated rats)

The effect of incubation time and protein concentration on the conversion of D_4 to metabolites by liver microsomes from PB-treated rats was determined. [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was incubated with a single concentration of liver microsomes from PB-treated Sprague-Dawley rats (0.25 mg protein/mL) for 0, 15, 30 and 60 min. In addition, [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was incubated with three concentrations of liver microsomes from PB-treated rats (0.125, 0.25 and 0.5 mg protein/mL) for 30 min. No-NADPH, zero-time and no-protein samples served as blanks. The remaining procedure was essentially the same as that described in the section of Preliminary Incubations.

Rat liver microsomes: comparison between control and PB-treated rats

Two concentrations of [^{14}C]-D_4 (3 and 5 μM, 49.5 and 82.5 nCi/incubation, respectively) were incubated with a single concentration of liver microsomes from PB-treated rats and saline-treated (control) rats (0.25 mg protein/mL) for 5 min to evaluate the difference in these two test systems. In addition, [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was incubated with a single concentration of liver microsomes from saline-treated rats (0.25 mg protein/mL) for multiple time periods (0, 5, 10 and 20 min) and with three concentrations (0.125, 0.25 and 0.5 mg protein/mL) for a fixed time period of 10 min. No-NADPH, zero-time and no-protein samples served as blanks. The remaining procedure was essentially the same as that described in the section of Preliminary Incubations.

Species comparison

[^{14}C]-D_4 was incubated with liver microsomes from human, PB-treated rats and saline-treated rats. Briefly, [^{14}C]-D_4 (5 μM, 82.5 nCi/incubation) was incubated with a single concentration of human liver microsomes (0.5 mg protein/mL) for multiple time periods (0, 15, 30 and 60 min) and with multiple concentrations (0.25, 0.5 and 1.0 mg protein/mL) for a fixed period of 30 min. In addition, [^{14}C]-D_4 (5 μM, 82.5 nCi/incubation) was incubated with a single concentration of liver microsomes from PB-treated rats or saline-treated rats (0.25 mg protein/mL) for multiple time periods (0, 5, 10 and 20 min) and with multiple concentrations (0, 0.125, 0.25 and 0.5 mg protein/mL) for a fixed time period of 10 min. The remaining procedure was essentially the same as that described in the section of Preliminary Incubations.

Metabolism of [^{14}C]-D_4 by recombinant human cytochrome P450 (CYP) enzymes

Microsomes from baculovirus-infected insect cell lines (Supersomes™) containing recombinant human CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9*1(Arg_{144}), CYP2C18, CYP2C19, CYP2D6*1(Val_{374}), CYP2E1, CYP3A4, CYP3A5 and CYP4A11 were purchased from Gentest Corporation (Woburn, MA). These recombinant human CYP enzymes (80-200 pmol of P450/incubation, 0.31-2.33 mg protein/mL) were incubated for 30 min in a 800-μL incubation volume with [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation). Microsomes (0.63 mg protein/mL) from baculovirus-infected insect cells containing empty vector were included in the assay as a negative control. The remaining procedure was essentially the same as that described in the section of Preliminary Incubations.

Based on the results of the experiment described above, an additional experiment was carried out with specific CYP enzymes to assess proportionality of metabolite formation with respect to incubation time and the amount of enzyme per incubation. Briefly, [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was incubated with a single concentration of CYP2B6 or CYP3A4 (100 pmol/incubation) for 0, 30, 60, 90 and 120 min and with multiple concentrations (0, 25, 50, 75 and 100 pmol/incubation) for 60 min.

Antibody inhibition

A single concentration of human liver microsomes (10 mg protein/mL) was preincubated for 15 min with inhibitory polyclonal antibodies against CYP2B6 or CYP3A4 at room temperature. These antibodies were raised in New Zealand White rabbits against purified rat CYP2B1/2 and CYP3A1/1, respectively. The ratio of the antibody to the microsomes was 100 μL of the serum (antibody) to 40 μL of microsomes at 10 mg protein/mL (25 μL serum/0.1 mg human liver microsomal protein). After preincubation, a buffer mixture containing potassium phosphate (50 mM, pH 7.4), magnesium chloride (3 mM), EDTA (1 mM, pH 7.4), and [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was added. The reactions were started by the addition of β-NADPH and incubated at 37±1°C for 60 min. Control incubations contained equal amounts of IgG purified

from preimmune rabbit serum. The remaining procedure was essentially the same as that described in the section of Preliminary Incubations.

Data processing

Data were processed with a spreadsheet computer program Microsoft Excel (Microsoft Corp., Seattle, WA). Linear regression analysis was performed with statistical weighting using a computer software program, GraFit (Version 4.09, Erithacus Software Limited, London, UK).

RESULTS AND DISCUSSION

HPLC Method development, preliminary evaluations and method validation

HPLC method was transferred from the Sponsor to the Testing Facility. Preliminary evaluations were carried out to establish typical experimental procedures (*e.g.*, order of adding reagents, volume of sample transfer). Due to the volatility of [^{14}C]-D_4, it was necessary to minimize exposure of D_4 to air. Additionally, D_4 forms a coat on glass surfaces; therefore, care was taken to avoid glass surfaces. Plastic pipets and vials (12 x 32 mm) were used throughout the study.

The HPLC method used in this study was qualified based on the criteria described the validation report (**Appendix 4**). In brief, the method was subjected to three-day validation experiments designed to ascertain the linearity of the detector response, precision and accuracy of the method, analyte stability and robustness of the method. The results of these experiments are detailed in **Appendix 4**. Calibration samples (zero-time incubations) were prepared fresh and analyzed for each experiment. Based on these results, purity of the test article did not change significantly from the original purity (99.67%) throughout the study.

Time and Protein-1 (Human liver microsomes)

Under the experimental conditions examined (incubation with 1 mg protein/mL for 60 min), [^{14}C]-D_4 was converted to one major metabolite by human liver microsomes. The retention times of the metabolite and [^{14}C]-D_4 were approximately 45 and 50 min, respectively (**Figure 2**). This metabolite peak was initially designated as M1. However, it was subsequently renamed M8 due to the results of incubations of [^{14}C]-D_4 with liver microsomes from PB-treated rats (discussed later). No metabolite formation was observed in zero-time, no-protein and no-NADPH incubations. However, the formation of metabolite M8 was not proportional to protein concentration or incubation time (**Figure 3**). Formation of M8 increased with respect to substrate concentration but it was not proportional to incubation time at any substrate concentration. As shown in **Table 1** and **Figure 4**, although up to 34% of the substrate was lost during the course of a 120-min incubation, the percentage of substrate converted to M8 did not exceed 10%.

Protein suitability and Time and Protein-2 (Human liver microsomes)

To establish the initial rate conditions for the metabolism of [^{14}C]-D_4, the "Time and Protein" experiment was repeated with lower protein concentrations and shorter incubation times. Microsomes were required in the substrate solution for [^{14}C]-D_4 to be solubilized and retained in solution. Thus, the solubility of D_4 in various concentrations of microsomal protein was assessed prior to the "Time and Protein-2" experiment. [^{14}C]-D_4 (0.4, 1.6 and 4 µM) solution was prepared in buffer solution in the presence of human liver microsomes (1 mg protein/mL and 0.05 mg protein/mL) and the percent recovery of [^{14}C]-D_4 in substrate solution was determined by measuring concentrations of [^{14}C]-D_4 present in the substrate solution by scintillation counting and comparing the observed and theoretical concentrations. At 1 mg

protein/mL, 92% of [^{14}C]-D_4 was recovered (average of the results at three substrate concentrations). However, at 0.05 mg protein/mL, the average recovery of [^{14}C]-D_4 was 68% (**Table 2**).

When [^{14}C]-D_4 was incubated for 15 min with various concentrations of microsomal protein (0, 0.01, 0.05, 0.1, 0.2, 0.5 and 1.0 mg protein/mL), detectable amounts of M8 were formed in incubations with 0.1 mg protein/incubation and above. However, the amount of M8 formed was not proportional to protein concentration (**Table 3** and **Figure 5**). The recovery of [^{14}C]-D_4 in incubations with varying concentrations of microsomal protein is shown in **Table 4**. As expected from the results of the protein suitability experiment described above, the actual substrate (D_4) concentration varied depending on the protein concentration in the substrate solution due to the solubility of D_4. For example, without any protein, the actual substrate concentration was 0.74 μM. On the other hand, in the presence of 0.5 mg protein/mL, the actual concentration was 2.39 μM, whereas in both cases the target concentration was 3 μM. However, approximately 70% of the substrate was solubilized in the incubation mixture at 0.2 mg protein/mL and above, and yet M8 formation was not proportional to incubation time.

When [^{14}C]-D_4 was incubated with human liver microsomes (0.1 mg protein/mL) for 1, 5, 10, 20, 30 and 60 min, M8 formation was observed in the samples incubated for 10 min or longer. However, the amount of M8 formed was not proportional to incubation time (**Figure 5**). Although the percent of D_4 converted to M8 did not exceed 7%, up to 53% of D_4 was lost during a course of 1 to 60-min incubation (**Table 5**). Based on the results of experiments described in "Time and Protein-1" and "Time and Protein-2", it was apparent that M8 formation was complex, and it would be difficult to establish initial rate conditions for this reaction because of the unusual physico-chemical properties of M8.

Assessment of binding of D_4 to human liver microsomes

The possibility that [^{14}C]-D_4 and its metabolite(s) bind to microsomal protein and that this was the cause of the loss of radioactivity in previous experiments was assessed. [^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was incubated with a single concentration of human liver microsomes (0.1 mg protein/mL) for 0, 20, 40 and 60 min (stepwise procedure is shown in **Figure 6**). As shown in **Table 6**, there was no significant difference in the radioactivity detected between the quenched incubation samples (samples containing THF to stop the reaction) and the supernatant fractions of the quenched incubation samples. In addition, the radioactivity in the protein pellet decreased as incubation time increased which suggests that the loss of radioactivity in the sample was not due to the binding of [^{14}C]-D_4 and its metabolite(s) to the microsomal protein. The percent recovery of the radioactivity in the substrate solution was 67% of the theoretical value, which decreased to approximately 40% after a 60-min incubation. The initial ~30% loss in radioactivity is likely due to the highly volatile nature of D_4. The additional ~30% loss following a 60-min incubation may be due to formation and loss of additional volatile

metabolites. (Note: the incubation volume was 800 µL in a 1-mL-size vial, thus, the headspace was 20% of the total volume of the container.) No further attempt was made to examine this possibility.

Time and protein (PB-treated rat liver microsomes) and rat liver microsomes comparison

Preliminary experiments were carried out to examine the metabolism of [^{14}C]-D_4 by liver microsomes from PB-treated rats and saline-treated (control) rats. The incubation conditions (*e.g.*, incubation time and protein concentration) were determined from these preliminary experiments (data not shown) and used for the species comparison experiment described below.

Species comparison

[^{14}C]-D_4 was incubated with liver microsomes from human, PB-treated rats or saline-treated male rats. In the presence of NADPH, liver microsomes from PB-treated rats converted D_4 to at least eight metabolites, which were named M1, M2, M3, M4, M5, M6, M7 and M8, based on their retention times (approximately 3.3, 6.4, 34.7, 35.4, 39.2, 40.0, 41.0 and 44.8 min, respectively), with M1 being the most polar and M8 the least polar (**Figure 7**). It should be noted that the peak designated M1 appeared to consist of multiple peaks. No attempt was made to resolve these peaks further. M8 was the major metabolite detected in incubations of [^{14}C]-D_4 with liver microsomes from saline-treated rats and human (**Figure 7**). In addition, metabolite M5 was detected in incubations with human liver microsomes at higher protein concentration (1.0 mg protein/mL) and in incubations with liver microsomes from saline-treated rats with longer incubation time (20 min) (**Figure 8**). No metabolites other than M8 and M5 were detected in incubations with human liver microsomes and liver microsomes from saline-treated rats. The extent to which [^{14}C]-D_4 is metabolized varied significantly among microsomes from human (~10%), saline-treated rats (~10%) and PB-treated rats (~80%). The percent of D_4 lost during a course of incubation (comparing between theoretical zero-time concentration and the actual concentration) varied among microsomes from human (31% in 30 min), saline-treated rats (33% in 10 min) and PB-treated rats (86% in 10 min) (data not shown). With human liver microsomes, M8 formation was not proportional to incubation time or protein concentration. With liver microsomes from saline-treated rats, M8 formation was not proportional to incubation time although it was proportional to protein concentration. With liver microsomes from PB-treated rats, a few metabolites showed proportionality between their formation and incubation time and/or protein concentration (*e.g.*, M3, M4, M7). It is notable, however, that M8 formation was not proportional to protein concentration or incubation time when D_4 was incubated with liver microsomes from PB-treated rats. This is probably due to exclusive consumption of the substrate and/or secondary metabolism of M8 by liver microsomes from PB-treated rats. These results suggest that CYP2B1/2 is involved in the metabolism of D_4 because treatment of rats with PB causes a marked (>20-fold) induction of CYP2B1 and CYP2B2.[6]

Metabolism of [^{14}C]-D_4 by recombinant human CYP enzymes

Representative HPLC chromatograms obtained from incubations of [^{14}C]-D_4 (3 μM) with recombinant human CYP enzymes are shown in **Figure 9**. In this experiment, recombinant CYP2B6 and CYP3A4 were the only two enzymes that catalyzed the formation of M8. The rate of M8 formation was approximately 4-fold higher with recombinant CYP2B6. In addition, M5 was detected only in incubations with recombinant CYP2B6. None of the other enzymes examined (including empty vector, which contains no human CYP activity) converted [^{14}C]-D_4 to detectable amounts of metabolite.

In order to make predictions about the contribution of CYP enzymes to the metabolism of a xenobiotic from data obtained from experiments with recombinant human CYP enzymes, the average specific content of these enzymes in human liver microsomes must be taken into account. The average percent of the total CYP per mg human liver microsomal protein for human CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 is available in the literature.[7] Based on this information, the rates of M8 formation (pmol/pmol P450/min) was normalized to reflect their activity in human liver microsomes, and the results are shown in **Table 7** and **Figure 10**. The results in **Table 7** suggest that CYP2B6 and CYP3A4 are largely responsible for converting [^{14}C]-D_4 to M8. The percent total normalized rate showing the contribution of CYP2B6 and CYP3A4 was 56% and 44%, respectively.

An additional experiment was carried out with CYP2B6 and CYP3A4 to assess proportionality of metabolite formation with respect to incubation time and the amount of enzyme per incubation. In the case of CYP2B6, the amount of M8 doubled when the concentration of CYP2B6 quadrupled. In all other cases, there was little or no relationship between M8 formation and either the concentration of CYP enzyme or incubation time (**Table 8** and **Figure 11**). Similarly, M5 formation was not entirely proportional to incubation time and amount of CYP2B6. Up to 37% of D_4 was lost in incubations with various amounts of CYP2B6 and CYP3A4. In addition, up to 66% of D_4 was lost during a course of 120-min incubation (**Table 9**).

Antibody inhibition

Formation of M8 by human liver microsomes was inhibited 32% and 34% by polyclonal antibodies against CYP2B and CYP3A, respectively (**Table 10** and **Figure 12**). The results are consistent with previous data suggesting that CYP2B6 and CYP3A4 are involved in the formation of M8. M5 formation could not be measured consistently in duplicate incubations, hence no data are shown for the effect of antibodies on its formation. Although the conversion of D_4 to M8 did not exceed 6%, the percentage of D_4 lost during 60-min incubations with human liver microsomes in the presence of anti CYP2B and anti CYP3A was 14% and 21%, respectively (**Table 11**).

CONCLUSIONS

In conclusion, [^{14}C]-D_4 was metabolized by human liver microsomes to one major metabolite (M8). Although the conversion of D_4 to M8 did not exceed 10%, the formation of M8 was not proportional to protein concentration or incubation time. The results of the experiment to assess [^{14}C]-D_4 binding to human liver microsomes suggest that the loss of radioactivity in the incubation sample was not due to the binding of [^{14}C]-D_4 and its metabolite(s) to the microsomal protein. Since D_4 is volatile, it is possible that one or more of its metabolites is volatile also, which would make it difficult to quantify. This is one possible explanation for the lack of proportionality of metabolite formation with incubation time and protein concentration. With human liver microsomes (and possibly liver microsomes from saline-treated rats), the results (lack of proportionality with incubation time and protein concentration) seem to suggest that only a portion of the added D_4 is available to be metabolized, which might occur if D_4 bound to plastic, formed micelles, or formed a film on the surface of incubation medium. However, the observation that with microsomes from phenobarbital-treated rats caused extensive metabolism of D_4 would seem to argue against such an interpretation of assay artifact and may suggest that microsomal metabolism of D_4 in the uninduced system is a complex blend of enzyme action and inhibition. Based on the results of experiments with recombinant human CYP enzymes and polyclonal antibodies, it was concluded that [^{14}C]-D_4 is primarily metabolized *in vitro* to M8, and that CYP2B6 and CYP3A4 are largely responsible for its formation.

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Table 1: A comparison of percent loss of substrate and percent of substrate converted to metabolites when D_4 (3 μM) is incubated with human liver microsomes

Substrate (μM)	Incubation time (min)	Percent loss of substrate (%)[a]	Percent of substrate converted to metabolites (%)[b]
3 μM, No NADPH	60	0	0
3 μM, + NADPH	15	7.0	9.7
	30	18.5	10.5
	45	19.3	9.3
	60	25.3	9.7
	120	34.4	10.0

[a]: (1-mean D_4 AUC in sample/mean D_4 AUC in blank) x 100

[b]: (Metabolite formed per incubation (pmol)/theoretical pmol of substrate per incubation) x 100

Blank: Values from "no-NADPH" sample were used as blank values

Theoretical pmol of substrate per incubation = 2400 pmol (3 μmol/L x 800 μL incubation volume)

See also Figure 4

Experimental title: Time and Protein-1 (Human Liver Microsomes)

Performed on 11/18/99

Table 2: Percent recovery of [^{14}C]-D_4 in the presence of various protein concentrations of human liver microsomes

Target Concentration of [^{14}C]-D_4 (μM)	Protein concentration (mg protein/mL)	Theoretical radioactivity (nCi)[a]	Actual radioactivity (nCi)[b]	Percent recovery (%)[c]
4	1	9.43	8.58	91.0
	0.05		6.67	70.8
1.6	1	3.77	3.55	94.2
	0.05		2.45	64.9
0.4	1	0.942	0.854	90.7
	0.05		0.646	68.6

[a]: Working solution (μM) x volume of aliquot of solution counted (0.0001 L) x specific activity (20.62 Ci/mol) x 1000. Values are rounded to three significant figures.

[b]: Mean of duplicate measurements (DPM)/2220 nCi/DPM. Values are rounded to three significant figures.

[c]: Actual radioactivity/theoretical radioactivity x 100 (%)

Experimental title: Protein suitability

Performed on 11/29/99

Table 3: Effect of incubation time and various microsomal protein concentrations on the conversion of D_4 (3 μM) to M8 by human liver microsomes

Protein concentration (mg protein/mL)	Incubation time (min)	M8 formed per incubation (pmol)[a]
0	15	ND
0.01	15	ND
0.05	15	ND
0.1	15	132
0.2	15	154
0.5	15	154
1.0	15	188
0.1	1	ND
0.1	5	ND
0.1	10	142
0.1	20	146
0.1	30	163
0.1	60	166

[a]: Mean of duplicate determinations

ND: not detected

See also Figure 5

Experimental title: Time and Protein-2 (Human Liver Microsomes)

Performed on 11/30/99

Table 4: Percent recovery of [^{14}C]-D_4 (3 μM) in substrate solution with various protein concentrations of human liver microsomes

Protein (mg /mL)	Theoretical radioactivity (nCi)[a]	Actual radioactivity (nCi)[b]	Actual concentration of [^{14}C]-D_4 (μM)[d]	Percent recovery (%)[c]
0	7.070	1.751	0.74	24.8
0.01	7.070	2.109	0.89	29.8
0.05	7.070	3.749	1.59	53.0
0.1	7.070	3.809	1.62	53.9
0.2	7.070	4.936	2.09	69.8
0.5	7.070	5.626	2.39	79.6
1.0	7.070	5.462	2.32	77.3

[a]: Working solution (μM) x volume of aliquot of solution counted (0.0001 L) x specific activity (20.62 Ci/mol) x 1000. Values are rounded to four significant figures.

[b]: Mean of duplicate determinations (DPM)/2220 nCi/DPM. Values are rounded to four significant figures.

[c]: Actual radioactivity/theoretical radioactivity x 100 (%). Values are rounded to three significant figures.

[d]: Target concentration of [^{14}C]-D_4 x Percent recovery/100 where the target concentration = 3 μM. Values are rounded to two decimal places.

Experimental title: Time and Protein-2 (Human liver microsomes)
Performed on 11/30/99

Table 5: A comparison of percent loss of substrate and percent of substrate converted to metabolites when D_4 (3 μM) is incubated with human liver microsomes

Substrate (μM)	Incubation time (min)	Percent loss of substrate (%)[a]	M8 formed per incubation (pmol)[b]	Percent of substrate converted to metabolites (%)[c]
3 μM, No NADPH	15	N/A	ND	0
3 μM, + NADPH	1	-3.5	ND	0
	5	5.5	ND	0
	10	11.2	142	5.9
	20	26.9	146	6.1
	30	25.0	163	6.8
	60	53.2	166	6.9

[a]: (1-mean D_4 AUC in sample/mean D_4 AUC in blank) x 100

[b]: Mean of duplicate determinations. Values are rounded to three significant figures.

[c]: (Metabolite formed per incubation (pmol)/theoretical pmol of substrate per incubation) x 100

N/A: Not Applicable

ND: Not detected

Blank: Values from "no-NADPH" sample were used as blank values

Theoretical pmol of substrate per incubation = 2400 pmol (3 μmol/L x 800 μL incubation volume)

Experimental title: Time and Protein-2 (Human liver microsomes)

Performed on 11/30/99

Table 6: Assessment of binding of D_4 (3 μM) to human liver microsomes

Sample[a]	Incubation time (min)	Theoretical activity (nCi)[b]	Actual activity (nCi)[c]	Percent recovery[d]
Substrate solution	0	7.07	4.74	67.0%
No THF	0	6.19	3.61	58.3%
THF		3.09	1.89	61.1%
Centrifuged		3.09	1.82	58.8%
Pellet		NA	0.08	NA
No THF	20	6.19	3.38	54.6%
THF		2.89	1.40	48.4%
Centrifuged		2.89	1.42	49.3%
Pellet		NA	0.05	NA
No THF	40	6.19	2.57	41.6%
THF		2.89	1.25	43.3%
Centrifuged		2.89	1.24	43.1%
Pellet		NA	0.04	NA
No THF	60	6.19	2.45	39.5%
THF		2.89	1.19	41.3%
Centrifuged		2.89	1.15	39.9%
Pellet		NA	0.04	NA

[a]: See Figure 6 for the detailed sample description.

[b]: Theoretical activity (values are rounded to two decimal places)

- Substrate solution = Concentration of D_4 working solution (3.429 μM) x volume of aliquot counted (100 μL) x specific activity (20.62 Ci/mol)
- "No THF" (Tetrahydrofuran) samples (zero-time and incubated)= Theoretical activity of Substrate solution x volume of substrate solution transferred (700 μL)/(volume transferred + NADPH, 800 μL)
- "THF" and "Centrifuged" = Concentration of D_4 working solution (3.429 μM) x volume of substrate solution transferred (350 μL)/(volume transferred + NADPH + THF, 800 μL) x volume of aliquot counted (100 μL) x specific activity (20.62 Ci/mol)
- Incubated (*e.g.*, T-20) THF and centrifuged samples = Concentration of D_4 working solution (3.429 μM) x volume of substrate solution transferred (700 μL)/(volume transferred + NADPH, 800 μL) x (volume transferred from incubation vial, 350 μL)/(volume transferred from incubation vial + volume of THF, 750 μL) x volume of aliquot counted (100 μL) x specific activity (20.62 Ci/mol)
- "Pellet" = NA (Not applicable)

[c]: Actual activity was determined by liquid scintillation counting (values are rounded to two decimal places)

[d]: Actual activity/theoretical activity x 100, determined for each fraction (values are rounded to one decimal place)

Experimental title: Assessment of binding of D_4 to HLM (Covalent binding experiment)

Performed on 2/24/00

Table 7a: Conversion of D_4 (3μM) to M5 by human CYP enzymes

CYP enzyme	Rate (pmol/min/pmol P450)	Nominal specific content (pmol P450/mg)[a]	Normalized rate (pmol/mg protein/min)[b]	Percent of total normalized rate[c]
CYP1A1	ND	0	ND	ND
CYP1A2	ND	45.0	ND	ND
CYP1B1	ND	ND	ND	ND
CYP2A6	ND	68.0	ND	ND
CYP2B6	0.0412	39.0	1.61	100%
CYP2C8	ND	64.0	ND	ND
CYP2C9	ND	96.0	ND	ND
CYP2C18	ND	ND	ND	ND
CYP2C19	ND	19.0	ND	ND
CYP2D6	ND	10.0	ND	ND
CYP2E1	ND	49.0	ND	ND
CYP3A4	ND	108	ND	ND
CYP3A5	ND	1.00	ND	ND
CYP4A11	ND	ND	ND	ND
		Total pmol CYP/mg[d]: 534	Total normalized rate: 1.61	

ND: Not determined

[a]: Data were obtained from Rodrigues, A.D. Integrated Cytochrome P450 reaction phenotyping: Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem. Pharmacol.* 57: 465-480, 1999.

[b]: Normalized rates were calculated by multiplying the rate (unrounded values) of formation of the metabolite of given recombinant CYP enzymes (in terms of pmol metabolite formed per pmol P450 present) by the nominal specific content. All values are rounded to three significant figures.

[c]: Calculated with Excel using unrounded values. All values are rounded to three significant figures.

[d]: Total CYP was measured spectroscopically as a ferrous-carbon monoxide complex.

See also Figure 10

Experimental title: Recombinant CYP enzymes

Performed on 1/12/00

(continued)

Table 7b: Conversion of D_4 (3μM) to M8 by human CYP enzymes

CYP enzyme	Rate (pmol/min/pmol P450)	Nominal specific content (pmol P450/mg)[a]	Normalized rate (pmol/mg protein/min)[b]	Percent of total normalized rate[c]
CYP1A1	ND	ND	ND	ND
CYP1A2	ND	45.0	ND	ND
CYP1B1	ND	ND	ND	ND
CYP2A6	ND	68.0	ND	ND
CYP2B6	0.0879	39.0	3.43	56.5%
CYP2C8	ND	64.0	ND	ND
CYP2C9	ND	96.0	ND	ND
CYP2C18	ND	ND	ND	ND
CYP2C19	ND	19.0	ND	ND
CYP2D6	ND	10.0	ND	ND
CYP2E1	ND	49.0	ND	ND
CYP3A4	0.0244	108	2.64	43.5%
CYP3A5	ND	1.00	ND	ND
CYP4A11	ND	ND	ND	ND
		Total pmol CYP/mg[d]: 534	Total normalized rate: 6.07	

ND: Not determined

[a]: Data were obtained from Rodrigues, A.D. Integrated Cytochrome P450 reaction phenotyping: Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem. Pharmacol.* 57: 465-480, 1999.

[b]: Normalized rates were calculated by multiplying the rate (unrounded values) of formation of the metabolite of given recombinant CYP enzymes (in terms of pmol metabolite formed per pmol P450 present) by the nominal specific content. All values are rounded to three significant figures.

[c]: Calculated with Excel using unrounded values. All values are rounded to three significant figures.

[d]: Total CYP was measured spectroscopically as a ferrous-carbon monoxide complex.

See also Figure 10

Experimental title: Recombinant CYP enzymes

Performed on 1/12/00

Table 8a: Effect of amount of CYP enzymes and incubation time on the conversion of D_4 (3 μM) to M5 by recombinant CYP2B6 and recombinant CYP3A4

Sample	P450 (pmol) incubation	Incubation time (min)	M5 formed per incubation (pmol)
CYP2B6	100	0	ND
		30	193
		60	213
		90	221
		120	244
	0	60	ND
	25		191
	50		216
	75		214
	100		236
CYP3A4	100	0	ND
		30	ND
		60	ND
		90	ND
		120	ND
	0	60	ND
	25		ND
	50		ND
	75		ND
	100		ND

ND: Not detected

Values for the amount of metabolite formed are rounded to three significant figures.

See also Figure 11

Experimental title: Time and protein with recombinant CYP enzymes

Performed on 7/26/00

(continued)

Table 8b: Effect of amount of CYP enzymes and incubation time on the conversion of D_4 (3 μM) to M8 by recombinant CYP2B6 and recombinant CYP3A4

Sample	P450 (pmol) incubation	Incubation time (min)	M8 formed per incubation (pmol)
CYP2B6	100	0	ND
		30	403
		60	414
		90	400
		120	389
	0	60	ND
	25		257
	50		330
	75		409
	100		493
CYP3A4	100	0	ND
		30	235
		60	248
		90	219
		120	213
	0	60	ND
	25		185
	50		219
	75		244
	100		208

ND: Not Detected

Values for the amount of metabolite formed are rounded to three significant figures.

See also Figure 11

Experimental title: Time and protein with recombinant CYP enzymes

Performed on 7/26/00

Table 9: A comparison of percent loss of substrate and percent of substrate converted to metabolites when D_4 (3 μM) is incubated with recombinant CYP2B6 and CYP3A4

Sample	Incubation time (min)	Amount of CYP enzyme (pmol/incubation)	Percent loss of substrate (%)[a]	Percent of substrate converted to metabolites (%)[b]
CYP2B6	0	100	N/A	0.0
	30		50.9	24.8
	60		64.7	26.1
	90		56.6	25.9
	120		65.9	26.4
	60	0	N/A	0.0
		25	18.7	18.7
		50	25.6	22.8
		75	34.2	26.0
		100	37.4	30.4
CYP3A4	0	100	N/A	0.0
	30		40.6	9.8
	60		44.9	10.3
	90		47.8	9.1
	120		46.1	8.9
	60	0	N/A	0.0
		25	34.8	7.7
		50	30.3	9.1
		75	24.5	10.2
		100	18.5	8.7

[a]: (1-mean D_4 AUC in sample/mean D_4 AUC in blank) x 100

[b]: (M5 + M8 formed per incubation (pmol)/theoretical pmol of substrate per incubation) x 100. (Values are rounded to once decimal place)

N/A: Not applicable

Blank = "Zero-time" sample for the time-course samples (0 to 120 min)

Blank = "no-cDNA (0 pmol) sample" for the protein-course samples (0 to 100 pmol)

Theoretical pmol of substrate per incubation = 2400 pmol (3 μmol/L x 800 μL incubation volume)

Experimental title: Time and protein with recombinant CYP enzymes

Performed on 7/26/00

Table 10: Antibody inhibition

M8 formation

Sample	M8 peak area[a]	Mean M8 formed per incubation (pmol)[b]	Percent control activity[c]	Percent inhibition[d]
Control IgG	22826	196	100.0%	0.0%
Control IgG	28173			
Anti CYP2B	11438	134	68.5%	31.5%
Anti CYP2B	12783			
Anti CYP3A	8761	130	66.3%	33.7%
Anti CYP3A	13598			

[a]: Value from HPLC chromatogram
[b]: Mean of duplicate determinations. Rounded to three significant figures.
[c]: (Mean metabolite formed per incubation/(Mean metabolite formed per incubation for Control IgG sample) x 100. Values are rounded to one decimal place.
[d]: 100-percent control activity. Values are rounded to one decimal place.

See also Figure 12

Experimental title: Antibody inhibition

Performed on 8/9/00

Table 11: A comparison of percent loss of substrate and percent of substrate converted to M8 when D_4 (3 μM) is incubated with human liver microsomes in the presence of antibodies

Sample	Percent loss of substrate (%)[a]	Mean M8 formed per incubation (pmol)[b]	Percent of substrate converted to M8 (%)[c]
Anti CYP2B, no-NADPH	N/A	0	0
Anti CYP2B + NADPH	14.2	134	5.6
Anti CYP3A, no-NADPH	N/A	0	0
Anti CYP3A + NADPH	21.2	130	5.4

[a]: (1-mean D_4 AUC in sample/mean D_4 AUC in blank) x 100 (values are rounded to one decimal place).
[b]: Mean of duplicate determinations. Rounded to three significant figures.
[c]: (Mean M8 formed per incubation (pmol)/theoretical pmol of substrate per incubation) x 100 (values are rounded to one decimal place).

N/A: Not applicable

Blank: Values from "no-NADPH" sample were used as blank values

Theoretical pmol of substrate per incubation = 2400 pmol (3 μmol/L x 800 μL incubation volume)

Experimental title: Antibody inhibition

Performed on 8/9/00

Figure 1: Structure of Octamethylcyclotetrasiloxane (D_4)

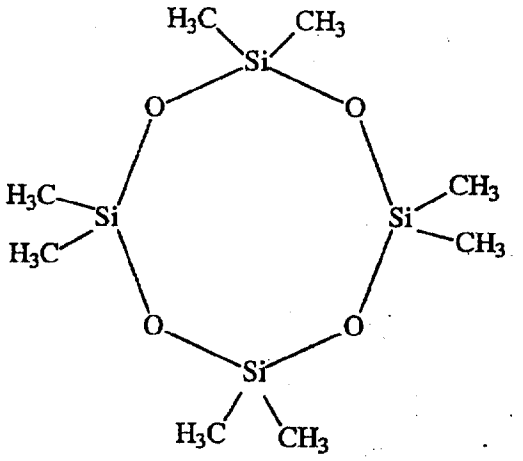
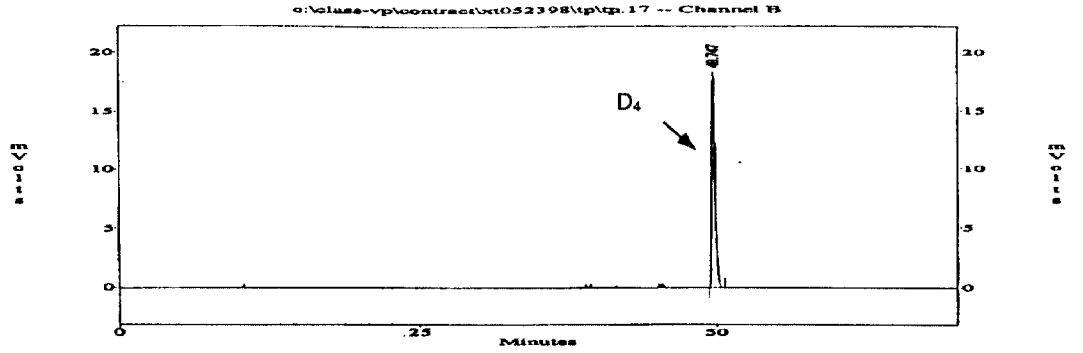
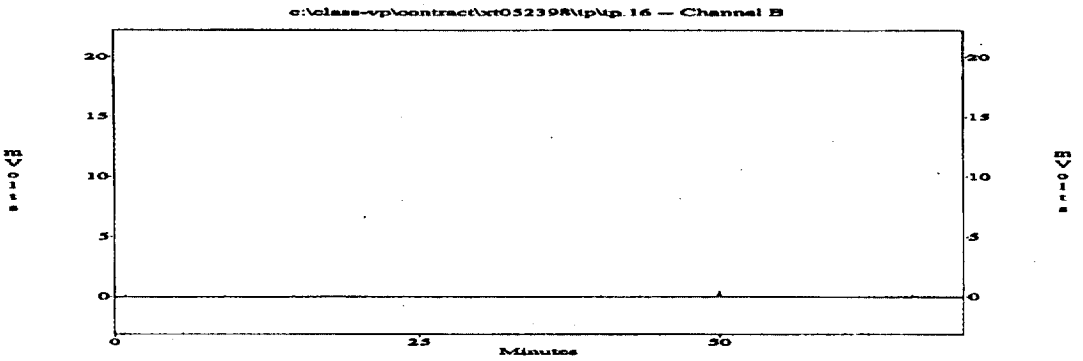


Figure 2: Representative HPLC chromatograms of [^{14}C]-D_4 (3 μM) incubations with human liver microsomes

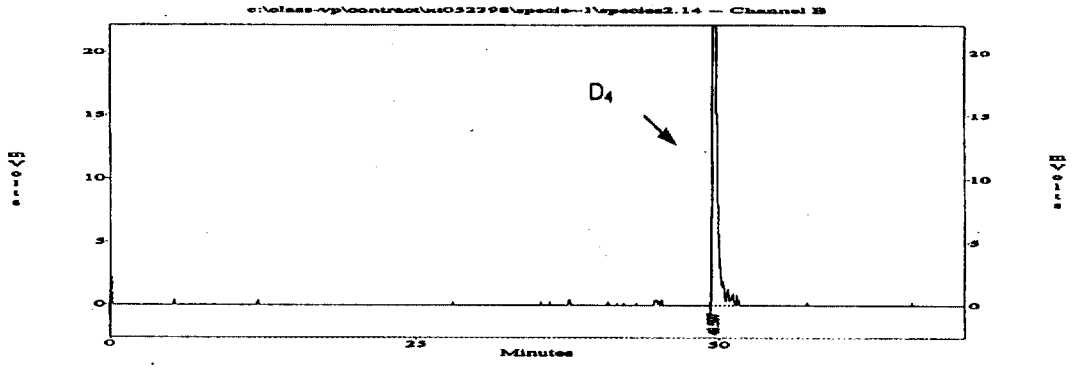
60-min incubation without NADPH



60-min incubation without protein



Zero-time incubation



60-min incubation with human liver microsomes at 1 mg protein/mL

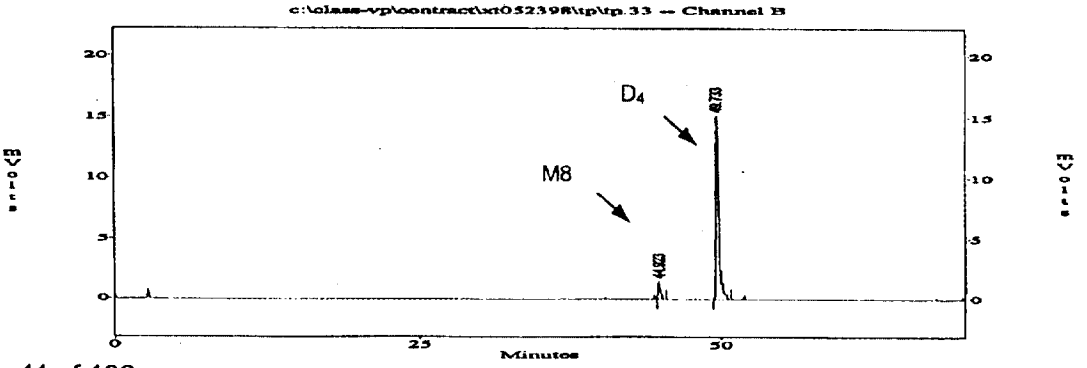
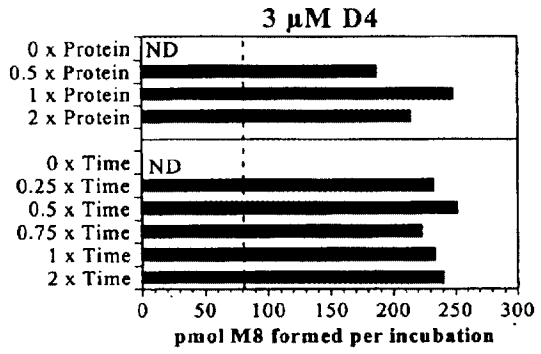
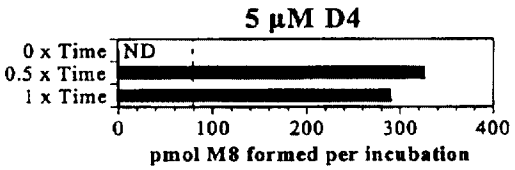
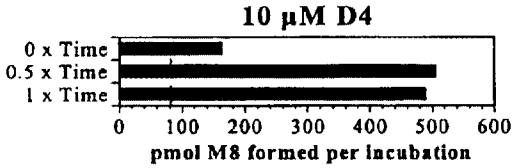


Figure 3: Effect of Time, Protein and Substrate concentration on the conversion of D_4 to M8 by human liver microsomes



1 x Protein = 1 mg/mL
1 x Time = 60 min





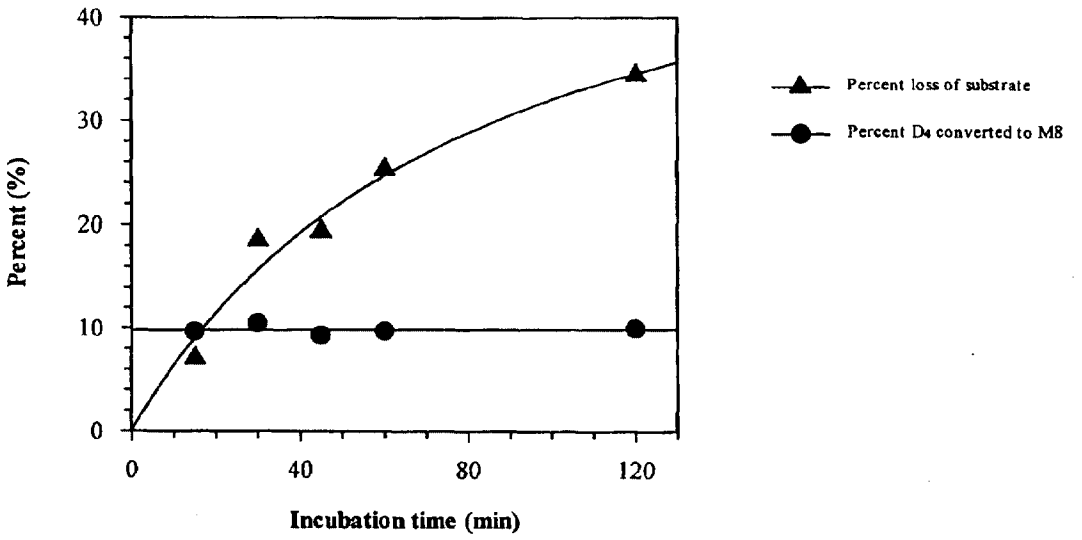
ND : Not detected

Dotted line represents the detection limit (peak area) set in the HPLC method. This limit corresponded to 84 pmol of analyte per incubation.

Experimental title: Time and Protein-1 (Human liver microsomes)

Performed on 11/18/99

Figure 4: A comparison of percent loss of substrate (D_4) and percentage of substrate converted to metabolites by human liver microsomes



Percent loss of substrate was calculated based on the assumption that the substrate AUC (mean of duplicate determinations) in the "no-NADPH" sample incubated for 60 min represents 100%

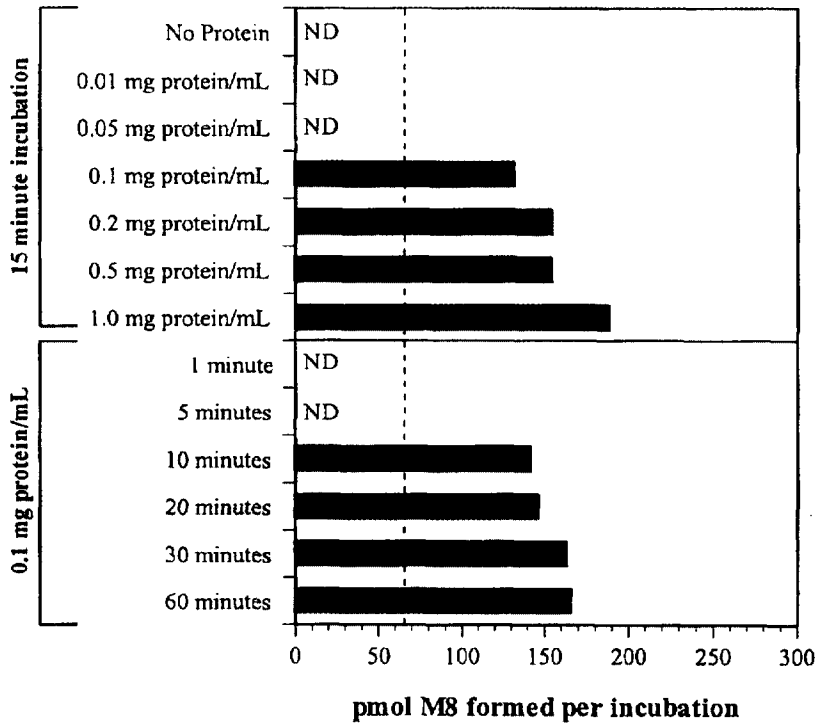
Percent of substrate converted to metabolite(s) = Percent of substrate converted to M8 = pmol of M8 formed/Theoretical amount of D_4 per incubation (pmol) x 100

Theoretical pmol of substrate per incubation = 2400 pmol (3 µmol/L x 800 µL incubation volume)

Experimental title: Time and Protein-1 (Human liver microsomes)

Performed on 11/18/99

Figure 5: Reevaluation of the effect of incubation time and protein concentration on the conversion of D_4 (3 μM) to M8 by human liver microsomes



ND: Not Detected

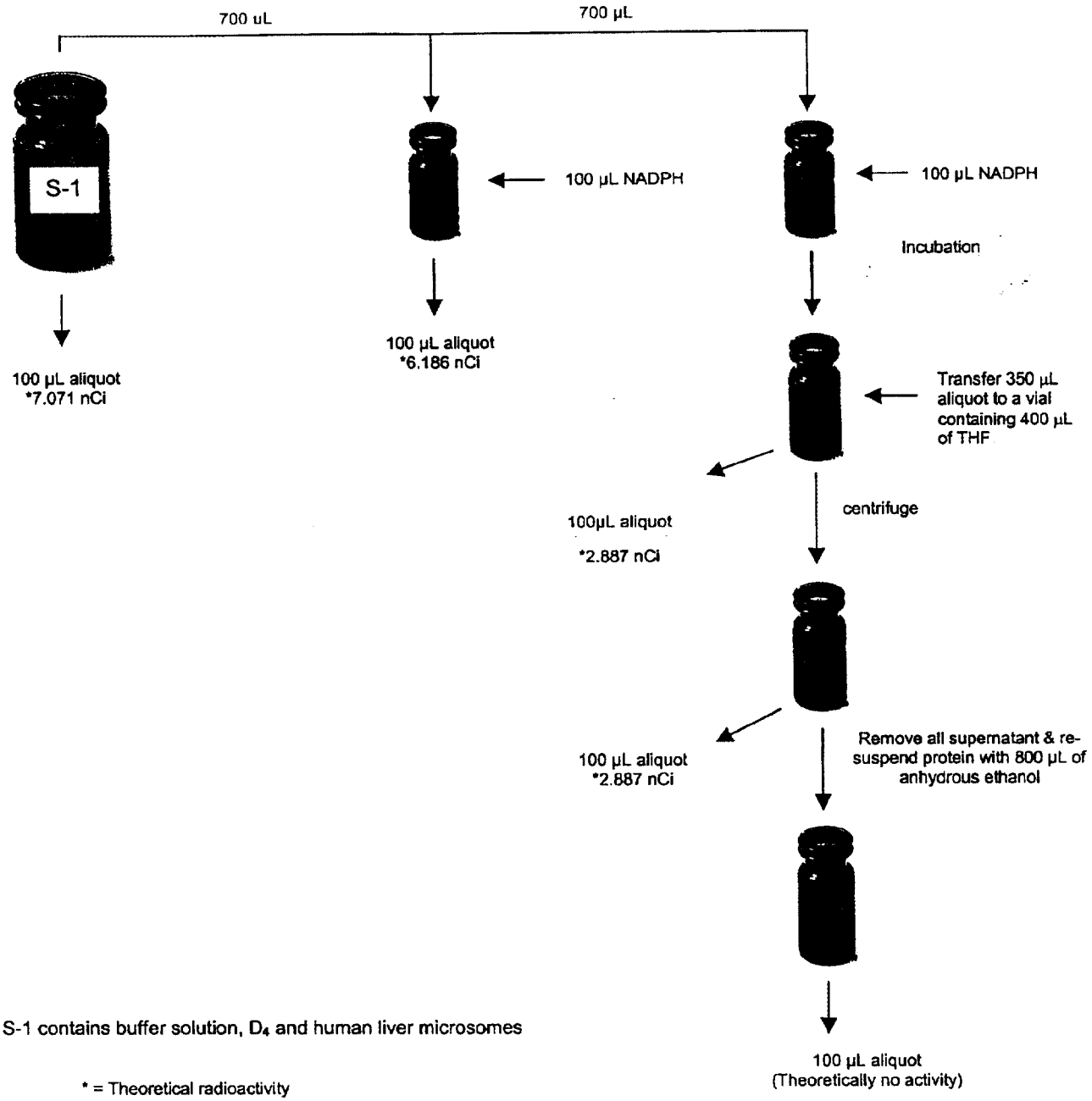
Dotted line represents the detection limit (peak area) set in the HPLC method. This limit corresponded to 67 pmol of analyte per incubation.

Experimental title: Time and Protein-2 (Human liver microsomes)

Performed on 11/30/99

Figure 6: Stepwise procedures for the Protein Binding Assessment

Incubated samples



S-1 contains buffer solution, D_4 and human liver microsomes

* = Theoretical radioactivity

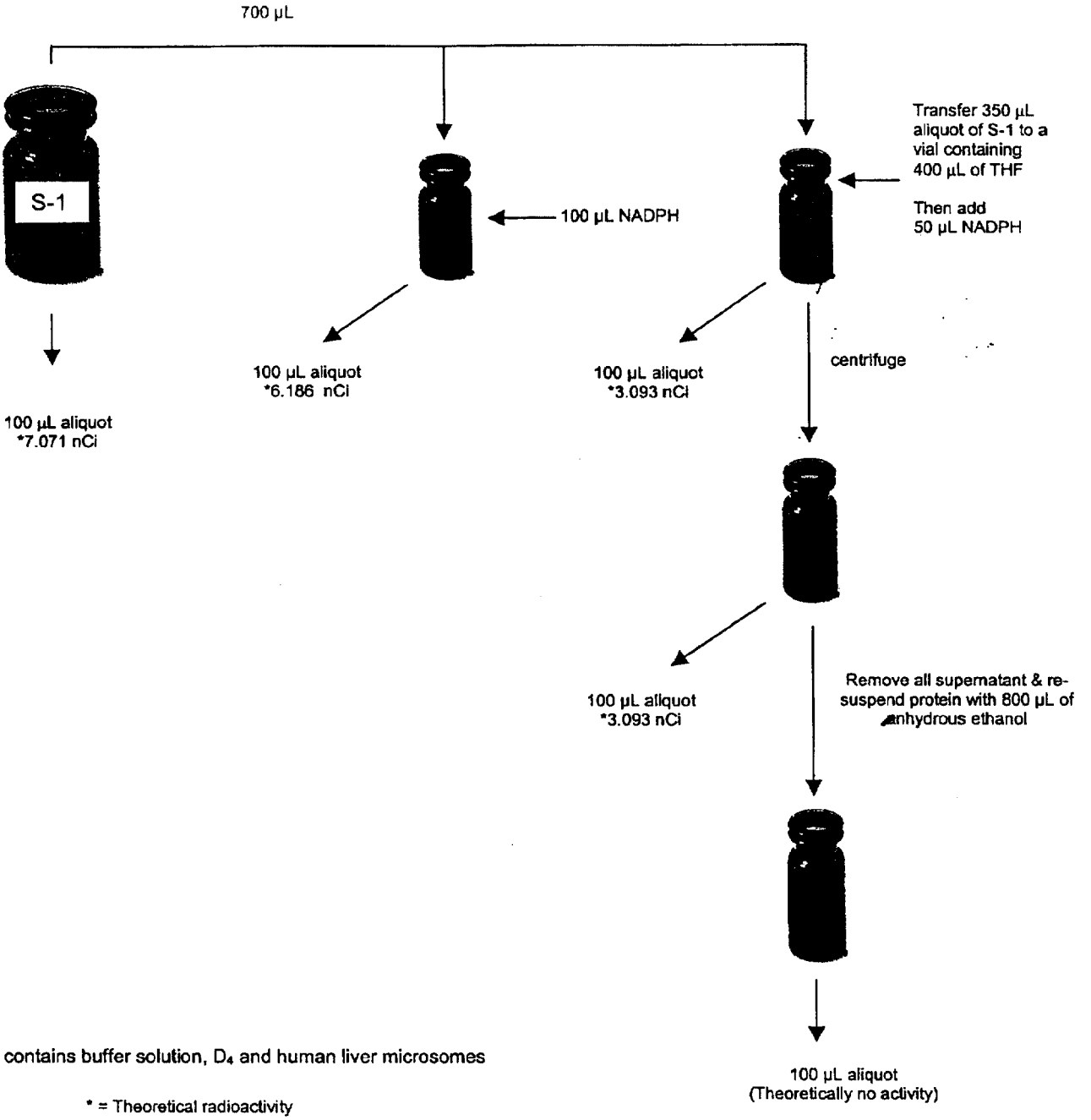
Experimental title: Assessment of binding of D_4 to HLM (Covalent binding experiment)

Performed on 2/24/00

(Continued)

(Figure 6 continued)

Zero-time samples (blank)



S-1 contains buffer solution, D_4 and human liver microsomes

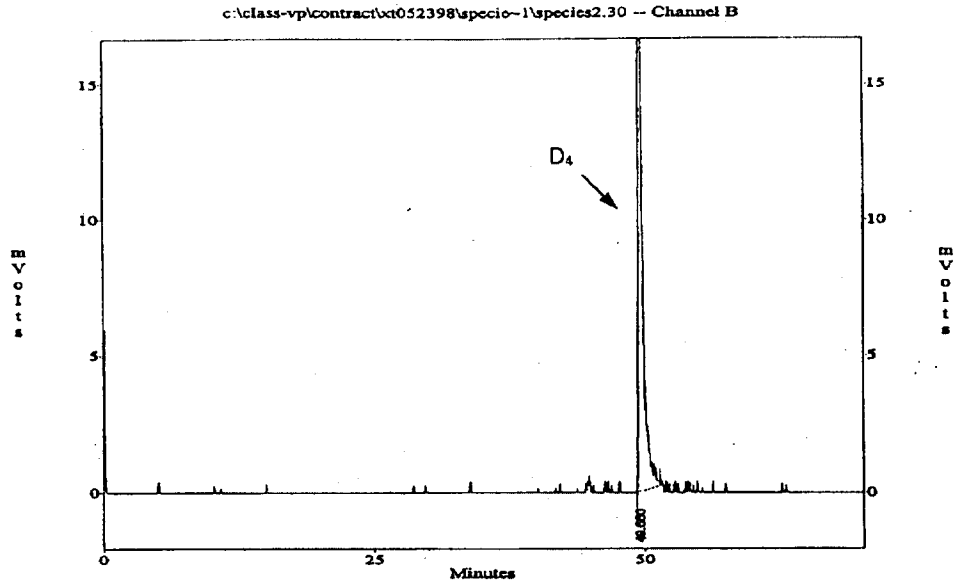
* = Theoretical radioactivity

Experimental title: Assessment of binding of D_4 to HLM (Covalent binding experiment)

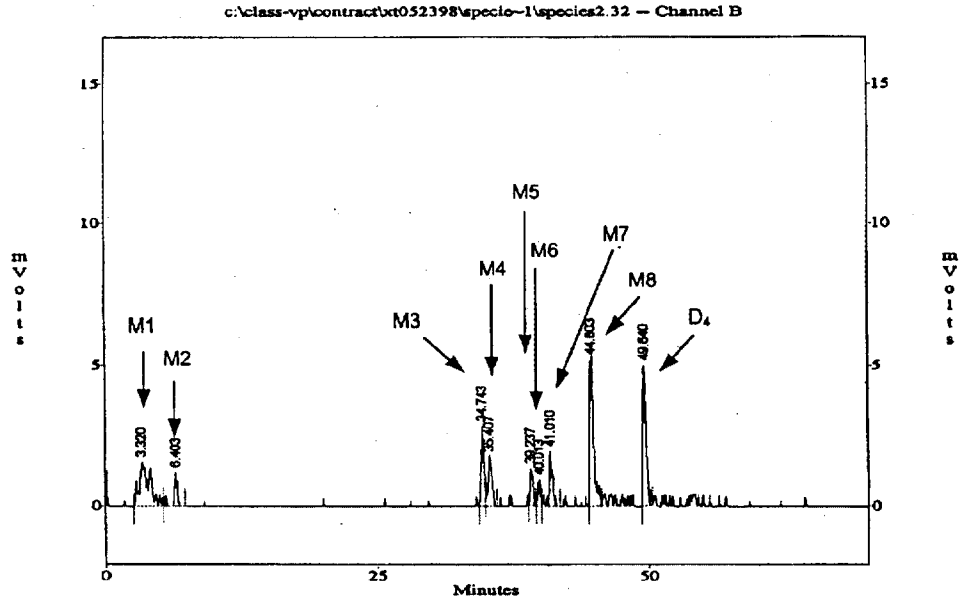
Performed on 2/24/00

Figure 7: Representative HPLC chromatograms obtained from "Species comparison-2"

Zero-time incubation with liver microsomes from PB-treated rats (0.25 mg protein/mL)



10-min incubation with liver microsomes from phenobarbital-treated rats (0.25 mg protein/mL)

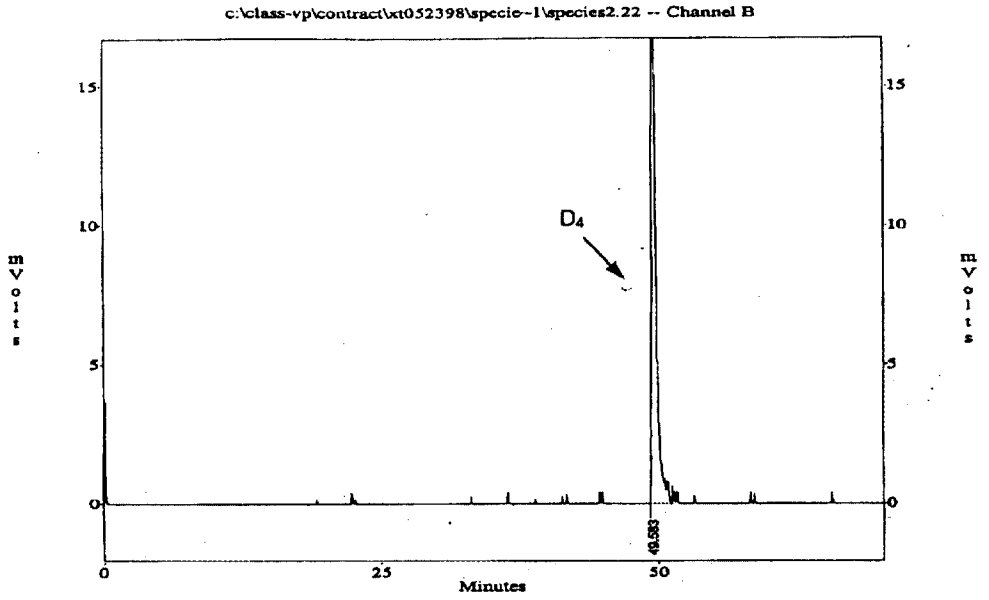


Experimental title: Species comparison-2, performed on 6/22/00

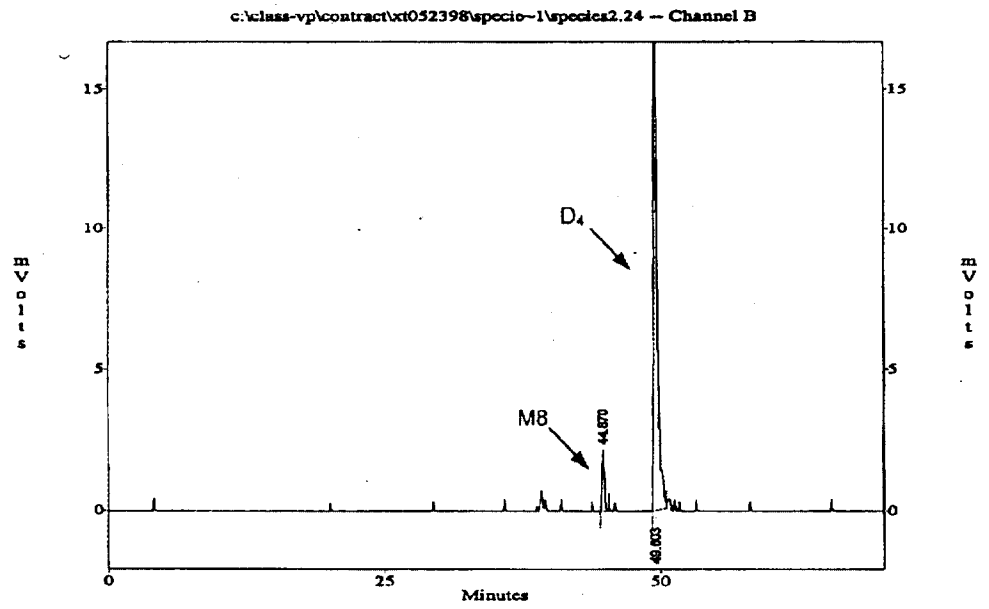
(Continued)

Figure 7 continued

Zero-time incubation with liver microsomes from saline-treated rats (0.25 mg protein/mL)



10-min incubation with liver microsomes from saline-treated rats (0.25 mg protein/mL)

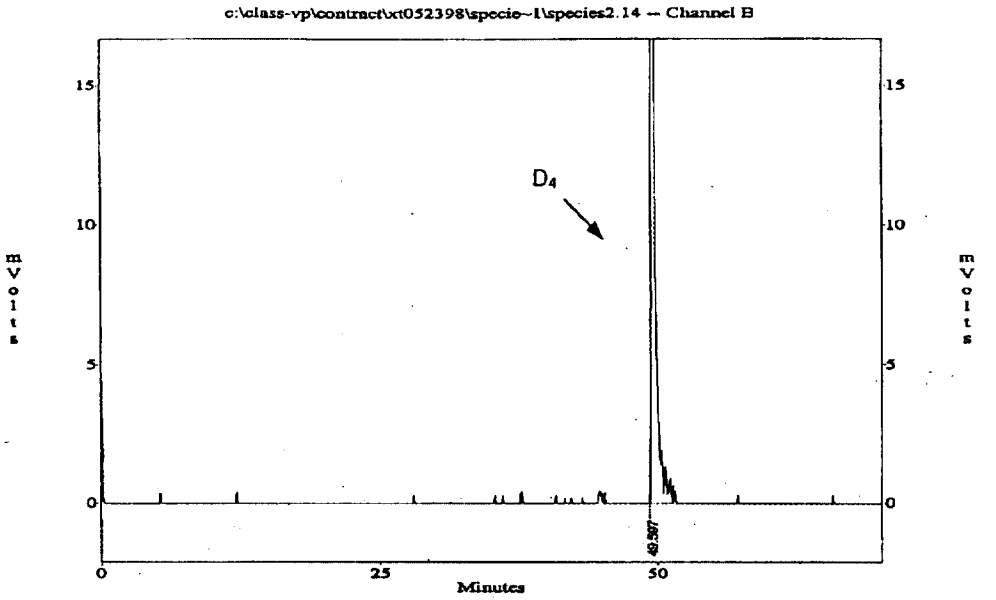


Experimental title: Species comparison-2, performed on 6/22/00

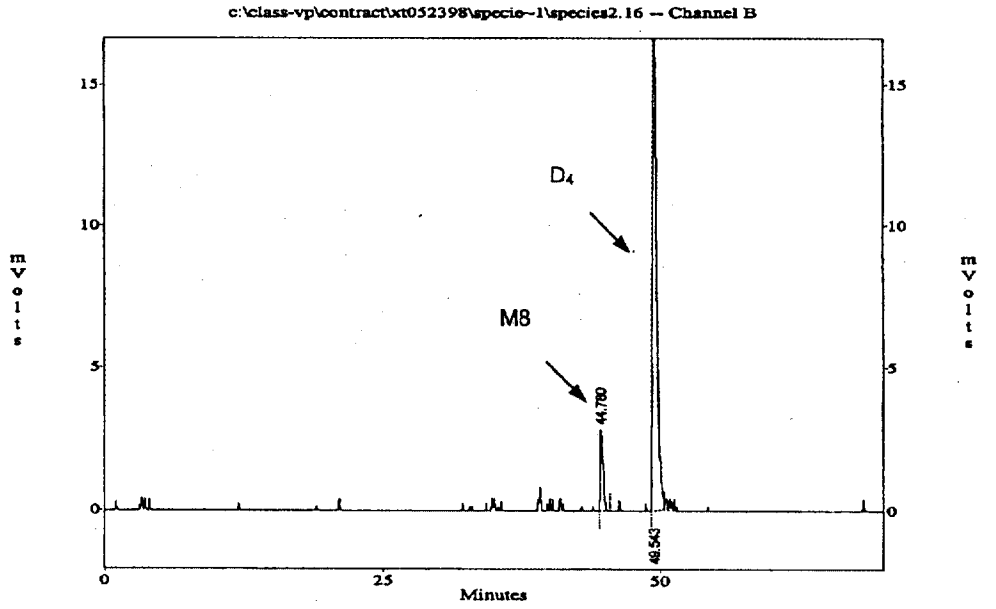
(continued)

Figure 7 continued

Zero-time incubation with human liver microsomes (0.5 mg protein/mL)



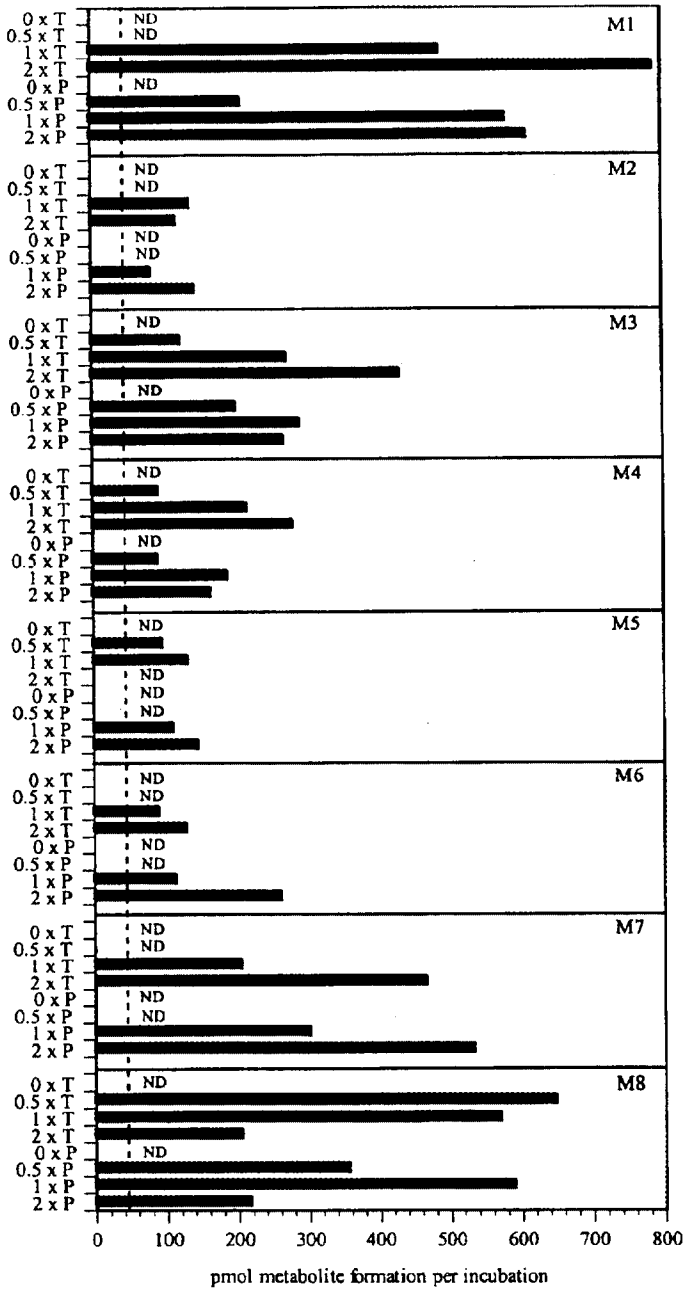
30-min incubation with human liver microsomes (0.5 mg protein/mL)



Experimental title: Species comparison-2, performed on 6/22/00

Figure 8: Effect of time and protein concentration

Incubation with liver microsomes from PB-treated rats



ND: Not detected: below the detection limit (peak area) set in the HPLC method. This limit corresponded to 42 pmol of analyte per incubation.

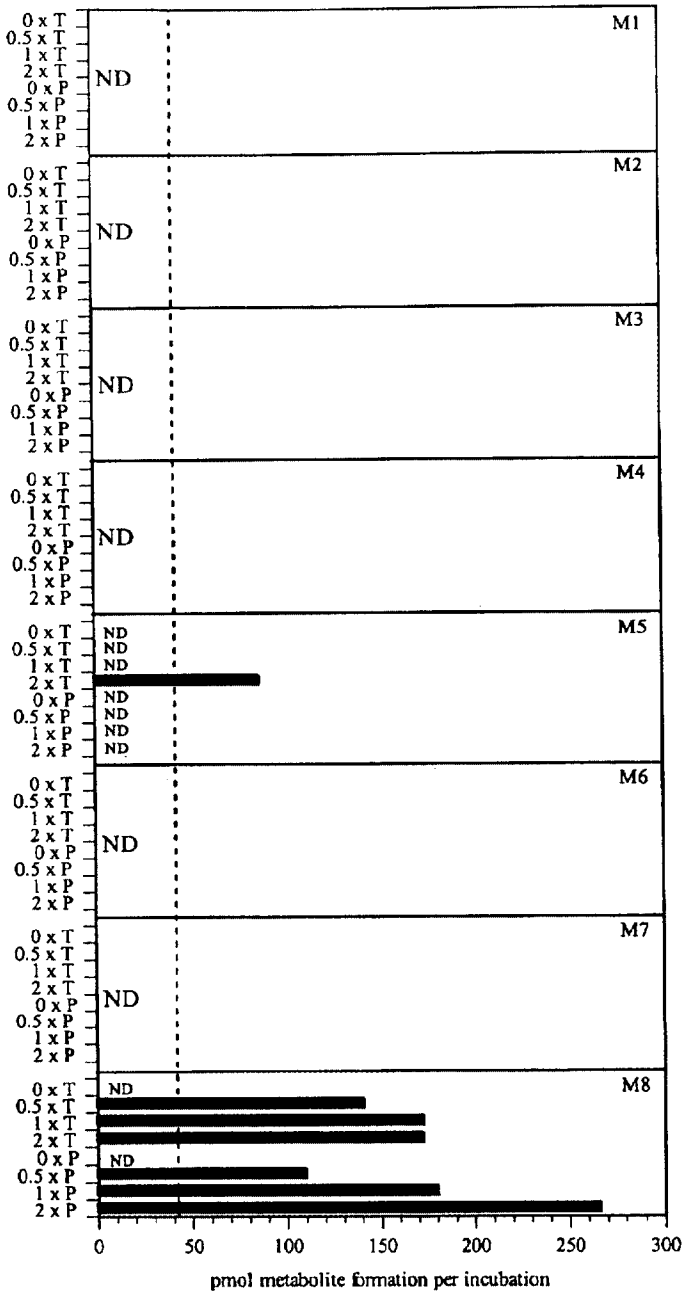
1 x T = 10 min

1 x P = 0.25 mg protein/mL

Experimental title: Species comparison-2, performed on 6/22/00

(continued)

Incubation with liver microsomes from saline-treated rats



ND: Not detected: below the detection limit (peak area) set in the HPLC method. This limit corresponded to 42 pmol of analyte per incubation.

1 x T = 10 min

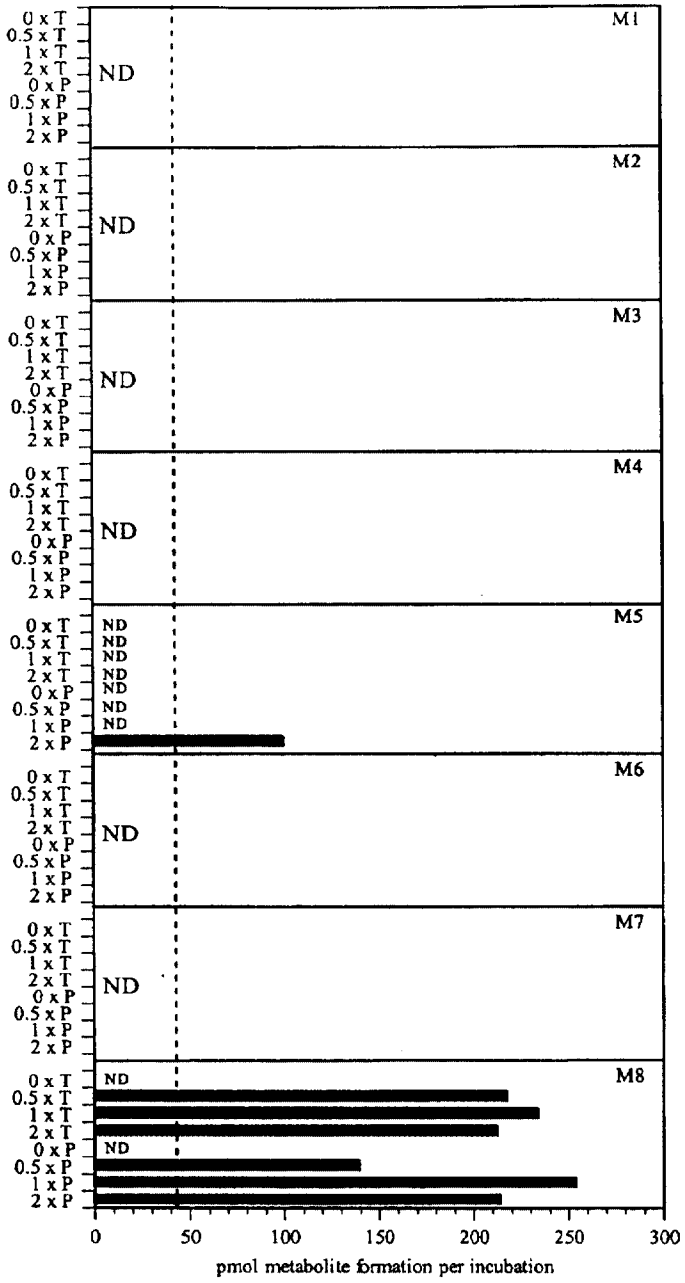
1 x P = 0.25 mg protein/mL

Experimental title: Species comparison-2, performed on 6/22/00

(continued)

Figure 8 continued

Incubation with human liver microsomes



ND: Not detected: below the detection limit (peak area) set in the HPLC method. This limit corresponded to 42 pmol of analyte per incubation.

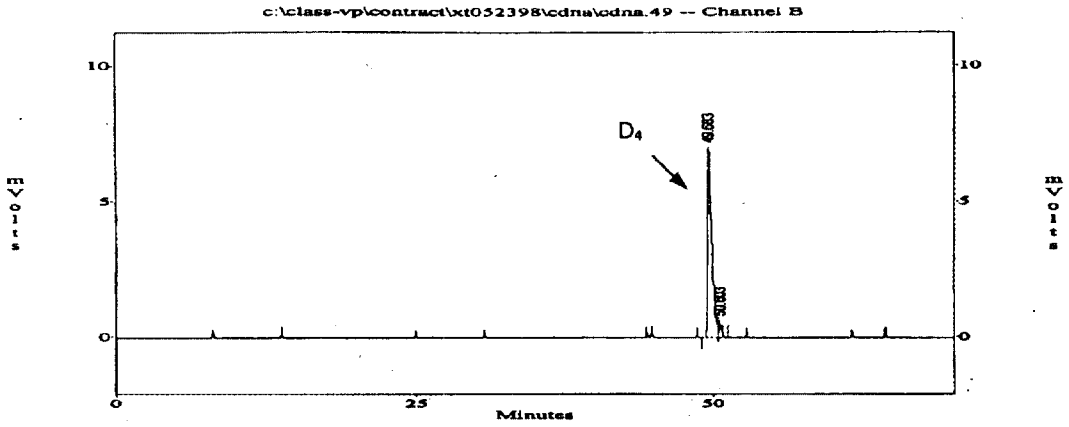
1 x T = 30 min

1 x P = 0.5 mg protein/mL

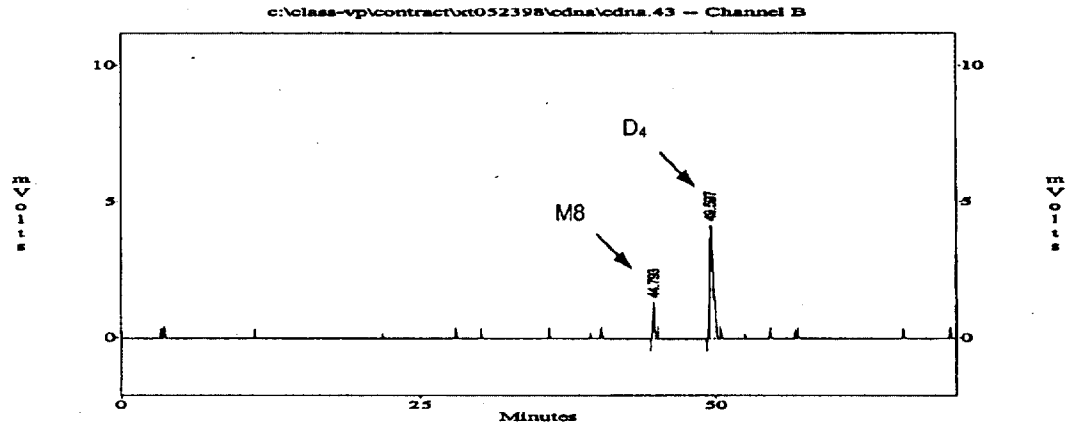
Experimental title: Species comparison-2, performed on 6/22/00

Figure 9: Representative HPLC chromatograms obtained from incubation of [^{14}C]-D_4 (3 μM) with recombinant CYP enzymes

30-min incubation with insect control



30-min incubation with CYP3A4 (200 pmol/incubation)



30-min incubation with CYP2B6 (100 pmol/incubation)

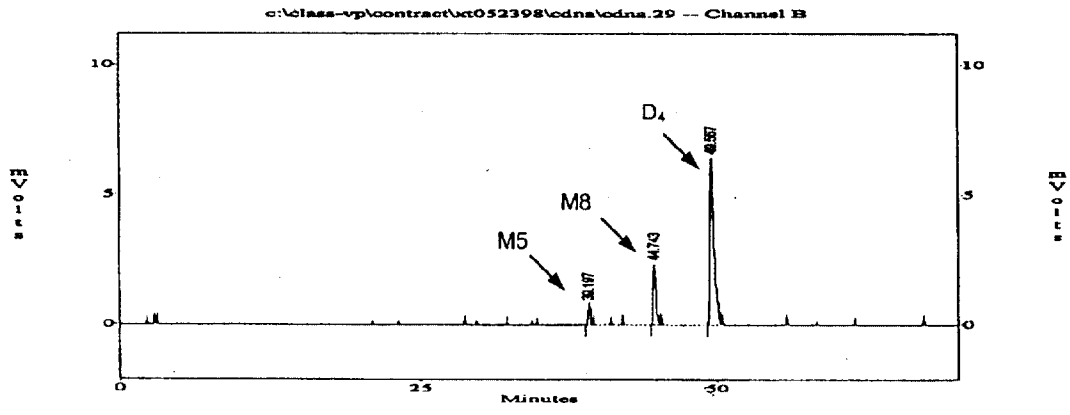
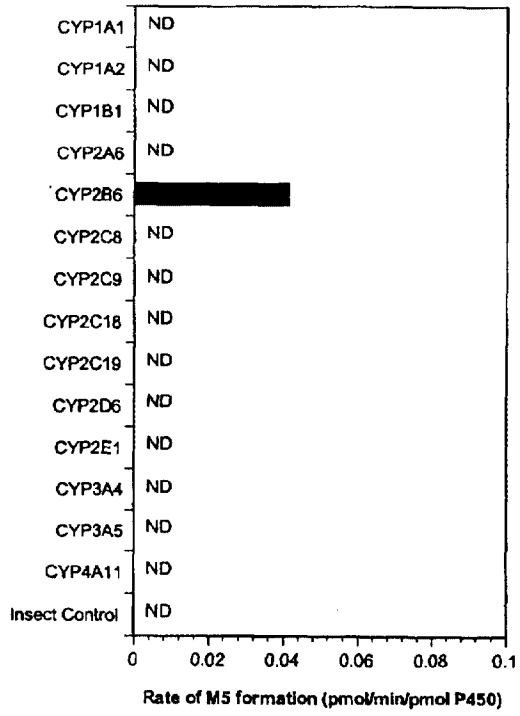
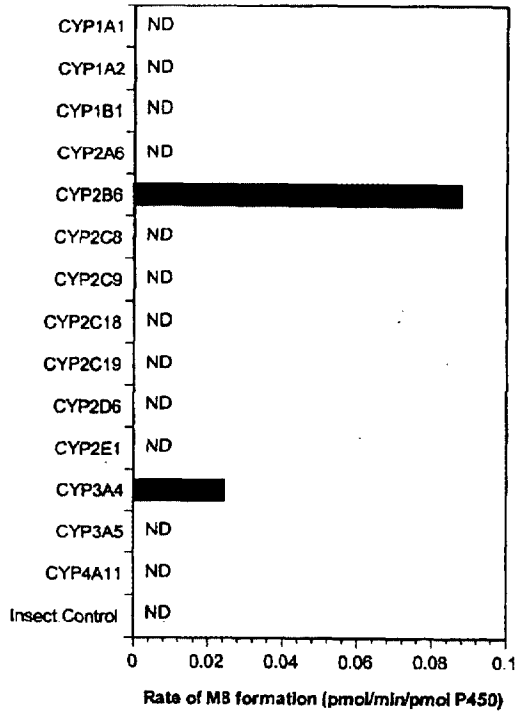


Figure 10: Rate of metabolite formation (incubation of D_4 with recombinant CYP enzymes)



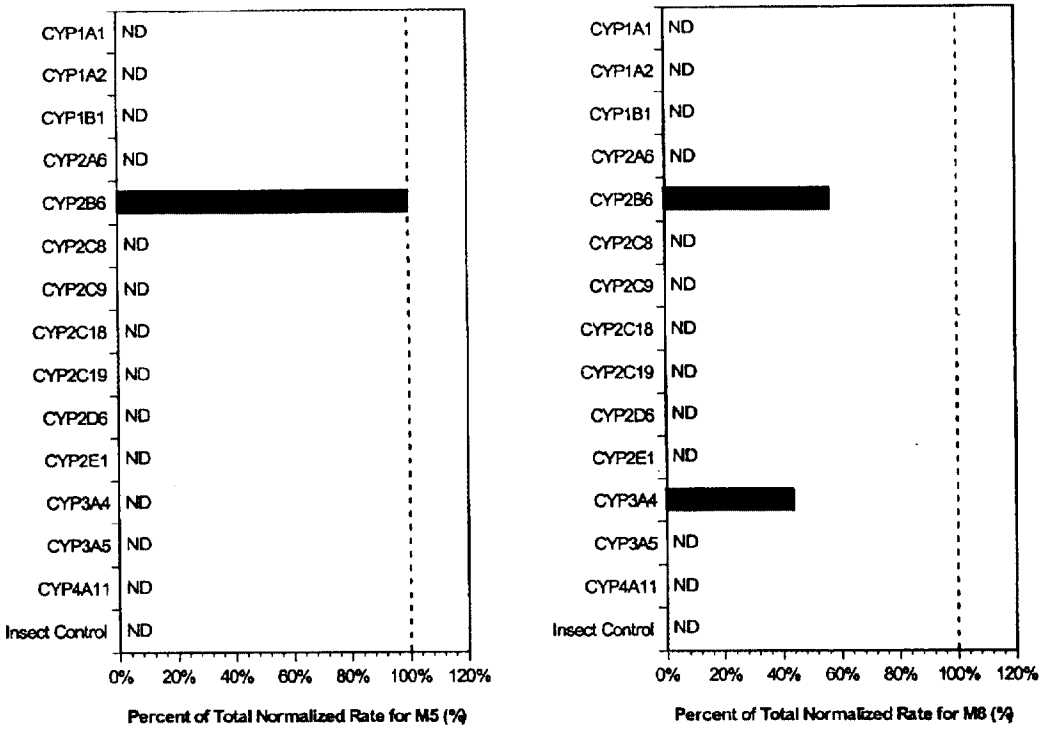


Experimental title: Recombinant CYP enzymes

Performed on 1/12/00

(continued)

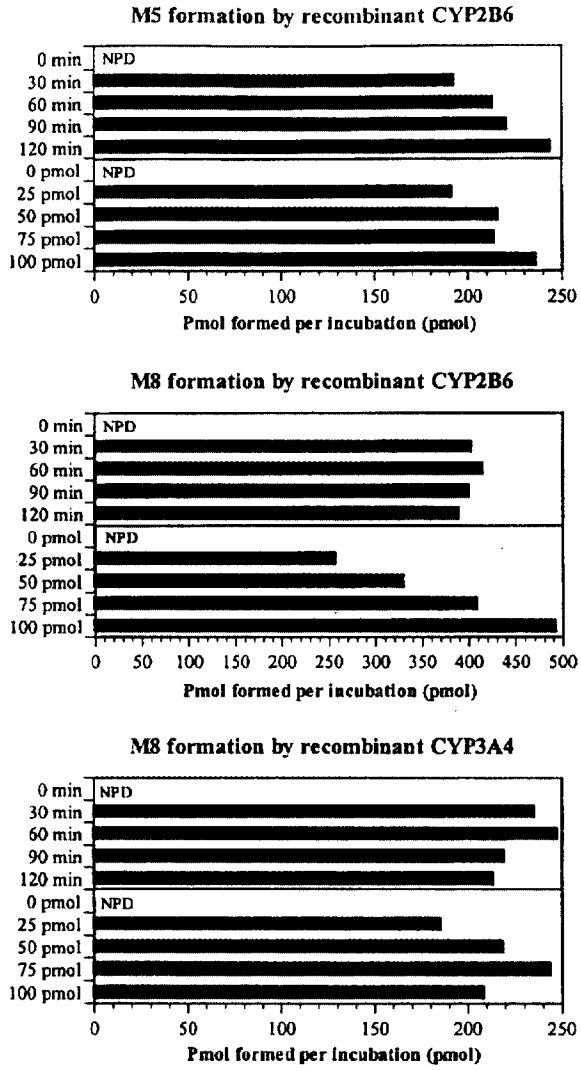
Figure 10 continued



Experimental title: Recombinant CYP enzymes

Performed on 1/12/00

Figure 11: Effect of incubation time and amount of P450 (incubations with recombinant CYP2B6 and CYP3A4)

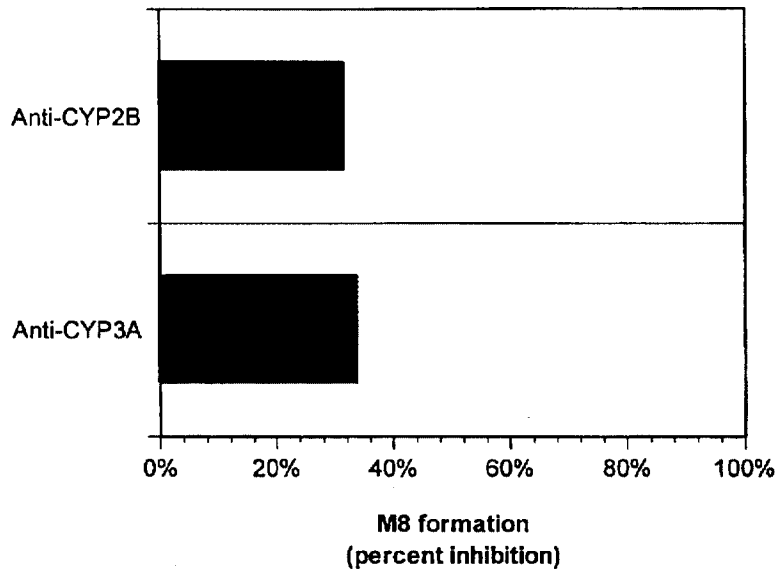


NPD: No peak detected

Experimental title: Time and Protein with recombinant CYPs

Performed on 7/26/00

Figure 12: Effect of selective antibodies on the conversion of [^{14}C]-D_4 to M8 by pooled human liver microsomes (n = 15)



Experimental title: Antibody inhibition

Performed on 8/9/00

Appendix 1: Certificate of analysis

Wizard Laboratories, Inc.

XT052398

DATE: 7/6/99

LOT NO.: 990316

Recd 7/9/99
SMB

PRODUCT INFORMATION

COMPOUND..... D4-^{14}C

ACTIVITY..... 1 mCi

SPECIFIC ACTIVITY..... 20.62 mCi/mmol

RADIOCHEMICAL PURITY... 99.67%

as determined by high-pressure liquid chromatography:

Column: C18, 5u, 4.6 x 250mm
Mobile Phase: ACN/H2O Gradient supplied by Dow Corning

Packaging: 1 x 1 mC. in sealed ampule under N2

J.E.D.

NOTE: The susceptibility of carbon-14 labeled compounds to radiolysis varies greatly. We urge you to use this product as soon as possible.

WARRANTY: Wizard Laboratories warrants this material to be as stated above. There is no warranty, expressed or implied, as to the fitness of this material for any particular purpose. The customer must notify the company, by registered mail, of any breach of warranty within 20 days of receipt.

2785-B DEL MONTE STREET WEST SACRAMENTO, CA 95691 USA TEL/FAX: (916) 372-2124 E-MAIL: SALES@WIZARDLABS.COM

Appendix 2: Characterization of ^{14}C-Octamethylcyclotetrasiloxane

DOW CORNING CORPORATION
HEALTH & ENVIRONMENTAL SCIENCES
TECHNICAL REPORT

Report No.:	1999-I0000-47184
Title:	Characterization of ^{14}C-Octamethylcyclotetrasiloxane (^{14}C-D_4, Lot # 990316)
Study No.:	9228
Test Article:	^{14}C-Octamethylcyclotetrasiloxane (^{14}C-D_4)
Study Director/Author:	Ying Sun
Sponsor:	Dow Corning Corporation 2200 W. Salzburg Road Auburn, MI 48611
Sponsor's Representative:	Roy A. Campbell Group Leader Toxicology Operations Group Dow Corning Corporation
Testing Facility:	Health and Environmental Sciences (HES) Dow Corning Corporation 2200 W. Salzburg Road Auburn, MI 48611
Study Completion Date:	07/07/99

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DC Study No. - 9228 DC Report No. - 1999-I0000-47184
Internal

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ABSTRACT

The test article, ^{14}C-octamethylcyclotetrasiloxane (^{14}C-D_4, Lot 990316), was characterized according to current EPA TSCA Good Laboratory Practice Standards. Hewlett Packard Gas Chromatography with mass spectrometer (GC/MS) detector was used qualitatively to aid in the identification of the major components in the test article. The radiochemical purity and specific activity of the test article, ^{14}C-octamethylcyclotetrasiloxane (^{14}C-D_4) were determined by High Performance Liquid Chromatography (HPLC) with a radioactivity flow-through detector and a Liquid Scintillation Counter (LSC).

Analysis by GC/MS verified the identity of the major component of the test article, ^{14}C-D_4, as radiolabeled octamethylcyclotetrasiloxane (D_4) which met the identification as defined in the MDMS. The average radiochemical purity for the test article, ^{14}C-D_4, was determined to be 99.563 ± 0.032 (area % ± standard deviation) which met the acceptance criteria for radiochemical purity. The mean specific activity determination of the replicate test article samples (9228-2 and 9228-3) did not meet the acceptance criteria; however, the specific activity of the test article 9228-3 (79.95 mCi/g or 23.67 mCi/mmol) was recommended for the use in future studies, as the specific activity for the test article, ^{14}C-octamethylcyclotetrasiloxane (^{14}C-D_4). The specific activity of this test article determined from this study will be used only as a guide and will be verified by study personnel prior to use in future studies.

DC Study No. - 9228

DC Report No. - 1999-I0000-47184
Internal

GLP COMPLIANCE STATEMENT

This study was conducted in accordance with the current EPA Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards, 40 CFR Part 792 except for the deviations listed in section I of this report.

Ying Sun
Senior Chemist
Study Director

07 July 99
Date

DC Study No. - 9228

DC Report No. - 1999-I0000-47184
Internal

QUALITY ASSURANCE STATEMENT

Title: Characterization of ^{14}C-Octamethylcyclotetrasiloxane (^{14}C-D_4, Lot # 990316)

Study Number: 9228

This study has been audited by the Dow Corning Corporation Health and Environmental Sciences Quality Assurance Unit according to approved Standard Operating Procedures to assure that the raw data are accurately reflected within this final report. The following are the inspection dates and the dates inspection findings were reported.

Dates of Inspection	Phase Inspected	Findings Reported to Study Director	Findings Reported to Management
01 Apr 99	Draft Protocol Review	01 Apr 99	07 Apr 99
09 Apr 99	Analytical Testing	09 Apr 99	13 Apr 99
21-22 Jun 99	Final Draft Report and Raw Data Review	01 Jul 99	06 Jul 99

Joyce J. Henry for Joyce Henry 06Jul99
Joyce J. Henry Date
Manager, Quality Assurance
Dow Corning Corporation
Health & Environmental Sciences

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APPROVAL SIGNATURES

This report consists of pages 1 through 22.

Ying Sun
Senior Chemist
Study Director

07 July 99
Date

Roy A. Campbell
Group Leader
Toxicology Operations Group
Sponsor's Representative

06-Jul-99
Date

DC Study No. - 9228 DC Report No. - 1999-I0000-47184
Internal

STUDY INFORMATION

Study Initiation Date:	04-07-99
Experimental Start Date:	04-08-99
Experimental Termination Date:	04-14-99
Study Completion Date:	07-07-99
Study Director:	Ying Sun Senior Chemist Test Article Characterization
Sponsor:	Dow Corning Corporation 2200 W. Salzburg Road Auburn, MI 48611
Sponsor's Representative:	Roy A. Campbell Group Leader Toxicology Operations Group
Study Personal:	Ying Sun Paul S. Larson

DC Study No. - 9228
DC Report No. - 1999-I0000-47184
Internal

A. Study Purpose/Justification for Selection of The Test System

The objective of this study was to verify the identity of the test article, and also determine the specific activity and radiochemical purity of the test article, ^{14}C-octamethylcyclotetrasiloxane (^{14}C-D_4), according to the current EPA Good Laboratory Practice Standards.

B. Test Article Information

Name:	^{14}C-Octamethylcyclotetrasiloxane (^{14}C-D_4)
Lot No.:	990316
Physical Description:	Colorless liquid, refer to MDMS and MSDS
Source:	Wizard Laboratories, Inc., 2785 B Del Monte St., W. Sacramento, CA 95691
Stability:	Will degrade if contacts with strong acids or bases. Radiolysis may occur in prolonged storage at high specific activity, according to the information provided by the sample submitter.
Radiochemical Purity:	99.67 area % ^{14}C-D_4, according to the information provided by Wizard Laboratories, Inc.
Specific Activity:	20.62 mCi/mmol, according to the information provided by Wizard Laboratories, Inc.
Expiration Date:	09/30/2001, according to the information provided by the sample submitter.
Solubility:	Soluble in tetrahydrofuran (THF), Hexane, Toluene and somewhat in acetonitrile (CH_3CN), according to the information provided by the sample submitter.
Storage Conditions:	Freezer, according to the information provided by the sample submitter.
Archive:	A reserve sample was not retained.

C. Route of Exposure and Justification

As the test article is the test system, this is not applicable.

D. Test System

^{14}C-Octamethylcyclotetrasiloxane (^{14}C-D_4)

E. Sample Labeling

The original test article container 9228-1 was labeled the following information: study number, name of test article, reference number, container accession number, concentration, storage conditions, expiration date, initials of the individual distributing and preparing the test article, and date of distribution and preparation. The container was also labeled as radioactive, with an estimated amount of radioactivity.

The original test article containers 9228-2 and 9228-3 were labeled the following information: study number, name of test article, lot number, container accession

number, concentration, storage conditions, expiration date, initials of the individual distributing the test article and date the test article was dispensed into the container. The containers were also labeled as radioactive, with an estimated amount of radioactivity.

Each diluted container and subsequent counting vial was labeled with a unique identifier and labeled as radioactive. All uniquely identified containers were cross referenced in the lab forms with a complete description as study number, name of material in the vials, unique identifier, concentration, expiration date and date of preparation.

F. Sample Preparation

Samples prepared for GC/MS (9228-1), HPLC (9228/HPLC) and diluted test article solutions (9228-2A and 9228-3A) were stored in a freezer.

GC/MS Solution

A wet needle amount of the test article, ^{14}C-D_4, was obtained in a 20 ml screw cap containing Teflon® coated septa vial which contained approximately 0.5 ml of hexane (as container 9228-1) for GC/MS analyses. After capping the vial, the contents were mixed well by shaking the vial several times by hand. A vial with only hexane solvent was prepared as a blank and was labeled as 9228/GCMS/B. The information for the hexane solvent used as a delivery substance was documented in the lab form.

LSC Counting Solutions

A 20 ml LSC vial with only approximate 14 ml scintillation cocktail was prepared as a blank and was labeled as 9228/LSC/B. Two replicate original test article samples (containers labeled as 9228-2 and 9228-3) were obtained in two 20 ml screw cap containing Teflon® coated septa vials. An approximate 5 ml of THF solvent was added to each containers (9228-2 and 9228-3) for the dilution of the test articles. The containers for the mixture of test article and solvent were labeled as 9228-2A and 9228-3A. The test article solution containers were mixed well by inverting each container several times by hand. For LSC counting, duplicate solutions (as containers 9228-2A-1 and 9228-2A- 2, 9228-3A-1 and 9228-3A-2) were prepared by placing 10 ul of the test article solutions, 9228-2A and 9228-3A, in separate 20 ml LSC vials which contained approximate 14 ml of compatible liquid scintillation cocktail. The information of the THF solvent as a delivery substance and cocktail used were documented in the lab forms. A verified balance was used to weigh the actual mass of the THF solvent and 10 ul of the test article solutions used.

HPLC Solution

An autosample vial with only approximate 1.5 ml THF solvent was prepared as blank and was labeled as 9228/HPLC/B. An HPLC solution was prepared by placing approximate 1.5 ml of the test article solution 9228-3A into an autosample vial for

HPLC analysis. The container of the HPLC solution was labeled as 9228/HPLC. The information of THF solvent used was documented in the lab form.

G. Experimental Design

1. GC/MS Analysis

A Hewlett Packard gas chromatography with mass spectrometer (GC/MS) detector was used to verify the identity of the major components in the test article as ^{14}C-octamethylcyclotetrasiloxane. Approximately 1.0 ul of hexane (9228/GCMS/B, as a blank) was manually injected prior to manually injecting approximately 1.0 ul of GC/MS solution 9228-1. The instrument conditions used were:

Oven Temperature Program:	50 °C for 3 minutes followed by a 15 °C/min. ramp to 280 °C for 4 min
Inlet Temperature:	250 °C
Capillary Column:	HP-5MS (Low Bleed 5%-Diphenyl-95% dimethylsiloxane copolymer), 0.25 mm inner diameter, 30 m length, 0.25 µm film thickness
Mode:	Constant flow, He carrier 2.2 psi at 50 °C, vacuum compensation on
Split Ratio:	~96.5:1
Mass Scan Range:	45-800 atomic mass units

Prior to test article analysis, the standard autotune procedure was conducted and the oven temperature was increased to approximately 280 °C for 40 min to prevent carry over by heating the column to burn off any residual material remaining from previous analysis. HP ChemStation version B.02.05 software and HP MS ChemStation version C.03.00 software were used for the data acquisition. The mass spectrum was library searched for the major component.

2. Specific Activity

A Packard 2500 TR liquid scintillation counter (LSC) was used to determine the specific activity. The personal computer software and instrument software used were version 1.03 and version 1.20. The LSC component identification was recorded in the raw data. The LSC quench curve (or calibration curve) was obtained by counting a series of Packard quenched ^{14}C LSC sealed standards. ^{14}C sealed standards were used daily when the instrument was in use to verify the accuracy of the quench curve.

A background vial (9228/LSC/B) with only approximate 14 ml cocktail and four sample solutions (9228-2A-1 and 9228-2A-2, 9228-3A-1 and 9228-3A-2) were counted. The samples were counted for 3 minutes and the background vial was counted for 10 minutes. The radioactivity was determined by the liquid scintillation counter (LSC). The specific activity were calculated by dividing the

total radioactivity by the weight of test article (in grams) determined gravimetrically (specific activity units = mCi/g).

3. **Radiochemical Purity**
The radiochemical purity was determined by Hewlett Packard high performance liquid chromatography (HPLC) with Packard series 500 radioactivity flow-through detector. The HPLC and radioactivity flow-through detector component identification were recorded in the raw data. A C_{18} 5-µm Alltech Altima column (250 mm length and 4.6 mm ID) was used for the HPLC analysis. The actual HPLC conditions used: 0-20 minutes, 100% H_2O at 1.0 ml/min; 20-40 minutes, linear gradient to 50:50 CH_3CN:THF at 1.0 ml/min; 40-50 minutes, 50:50 CH_3CN:THF at 1.0 ml/min; 50-55 minutes, linear gradient back to the initial conditions and 55-60 minutes, 100% H_2O at 1.0 ml/min. The actual detector conditions consisted of Ultima Flo-M as the scintillation cocktail, flow rate of 3 ml/min for 70 minutes and liquid flow cell size of 0.5 ml. The actual instrument conditions were documented in the raw data.

Triplicate injections (10 ul per injection) of the HPLC solution (9228/HPLC) prepared from the test article solution 9228-3A were made onto the HPLC system using the autosampler. Blank (THF, 9228/HPLC/B) injections (10 ul per injection) were made prior and after the HPLC solution injections. The "average value" background mode was used to determine background radioactivity. The background value was calculated over a specified time region (from 5.00 min to 10.00 min) of the chromatographic trace in which there are no peaks.

Determination of Test Article Recovery
The HPLC loop check was conducted before the HPLC analysis. Three injections of the HPLC solution (9228/HPLC) were made onto the HPLC loop (the connection between the loop and precolumn was disconnected before the loop check). The HPLC conditions used: 50:50 CH_3CN:THF at 1.0 mL/min for 3 minutes. A blank was collected using a 20 mL LSC vial before each injection. After each injection is started, three collections for the injection were taken using three 20 mL LSC vials for the first minute, second minute and third minute. Approximate 14 mL of scintillation cocktail was added to the four collection vials for each injection, then analyzed by liquid scintillation counting (Packard 2500 TR LSC). The sum of radioactivity count from the three collections for each injection was obtained. The average radioactivity count from the three injection determination was used for calculation of the % recovery.

The effluent from the third HPLC solution injection was collected in a tared 16 oz. glass jar. Approximate 14 mL of the effluent was weighed using a verified balance and placed separately in two 20 mL LSC vials then analyzed by liquid scintillation counting (Packard 2500 TR LSC) to determine the % recovery. The effluent from the blank injection (post HPLC solution injections) was collected in

a tared 16 oz. glass jar. Approximate 14 mL of the THF effluent was weighed using a verified balance and placed separately in two 20 mL LSC vials then also analyzed by liquid scintillation counting (Packard 2500 TR LSC). The results showed only insignificant radioactivity was detected in the post blank run.

H. Statistical Methods

Calculation of the average specific activity was not performed for ^{14}C-D_4 (see Deviation section). Calculation of the average area percent and standard deviation for radiochemical purity was performed for ^{14}C-D_4.

I. Deviations

A protocol deviation was written and included in the study file. The protocol states the detector (radioactivity) conditions will consist of Ultima Flo-M as the scintillation cocktail, flow rate of 3 ml/min for 60 minutes and liquid flow cell size of 0.5 ml. The actual run time for the HPLC analysis used was 70 minutes which was different from the parameters described in the protocol. Since the last 10 minutes of the run time was the equilibrium time with 100% H_2O for the system and the pump in the detector actually pumped for 70 minutes (70 min. run length was set up in the detector method), this deviation did not affect the quality or integrity of the data.

A protocol deviation was written and included in the study file. The section H. Statistical Methods in the protocol stated calculation of the average specific activity will be performed for ^{14}C-D_4. However, the calculation of the average specific activity was not performed for the test article (^{14}C-D_4). The reason was that the specific activity determined from the test article sample 9228-2 was unacceptable due to the unreliable weight of test article sample (0.00074 g) which was not in agreement with the volume of test article that the test article distributor intended to distribute. A note for explanation of the unreliable test article weight was written by the test article distributor and included in the study file. The specific activity determined from the test article sample 9228-3 (79.95 mCi/g or 23.67 mCi/mmol) will be used as a guide only since the specific activity of this test article will be verified by study personnel prior to use in future studies. Therefore, this deviation did not affect the quality or integrity of this study.

J. Results and Discussion

1. GC/MS Analysis

The identity of the test article (^{14}C-D_4) was verified as radiolabeled octamethylcyclotetrasiloxane by GC/MS analysis. The increased abundance of the following ions [where MW (D4) = 296]:

m/z 283 [$(M\text{-}CH_3)^+$ ion + 2 amu]
m/z 285 [$(M\text{-}CH_3)^+$ ion + 4 amu]
m/z 287 [$(M\text{-}CH_3)^+$ ion + 6 amu]

Table 1. Comparison of Abundance of Ion m/z 281 for Radiolabeled and Non-Radiolabeled Octamethylcyclotetrasiloxane (D_4)

Test Article ID	m/z $(M-CH_3)^+$ ion	Abundance	m/z $(M-CH_3)^+$ ion + 2 amu	Abundance	m/z $(M-CH_3)^+$ ion + 4 amu	Abundance	m/z $(M-CH_3)^+$ ion + 6 amu	Abundance
D_4 from Study # 8819 (lot# LL084732)[1]	281	10000	283	2046	285	140	287	1
Normalized to 100%	281	100	283	20	285	1	287	0
D_4 from Study # 8819 (lot# LL024S10)[1]	281	10000	283	2042	285	139	287	0
Normalized to 100%	281	100	283	20	285	1	287	0
D_4 from Study # 9176 (lot# LL098271)[2]	281	10000	283	2272	285	155	287	6
Normalized to 100%	281	100	283	23	285	2	287	0
^{14}C-D_4/Hexanes from Study # 9228 (Ref.# 9228-1)	281	100	283	53	285	11	287	1

is due to the presence of more than one ^{14}C labeled methyl group relative to that of non-radiolabeled octamethylcyclotetrasiloxane (See Table 1). The Total Ion Chromatogram (TIC) for the test article solution (9228-1) is shown in Figure 1. The mass spectrum of ^{14}C-D_4 is shown in Figure 2. The base peak at m/z 281 can be accounted for the lost of one -CH_3 group from the parent molecule[$(M\text{-}CH_3)^+$]. The ion m/z of 73 corresponds to Me_3Si^+ ion from the parent molecule. The four peaks at approximate 2.3, 2.4, 2.5 and 2.7 minutes shown in the TIC (Figure 1) were the solvent peaks in comparison with the TIC of solvent blank run. In addition, the mass spectrum was library searched for the major component. Unfortunately, no reasonable match is obtained since there were no radiolabeled octamethylcyclotetrasiloxane (^{14}C-D_4) files existed in the libraries. Even through, the identification of the test article (^{14}C-D_4) was successfully verified by the interpretation of its spectrum.

2. Specific Activity

The original test article samples (9228-2 and 9228-3) in separate 20 ml LSC vials were diluted with THF solvent (Identified as 9228-2A and 9228-3A) for the specific activity determination. The weights of test article samples and the total weight of diluted test article solutions are shown in Table 2.

Table 2. Sample Weight

Sample ID	Weight of Test Article Sample (g)	Weight of THF Used (g)	Weight of Test Article Solution (g)
9228-2A	0.00074	4.634	4.63474
9228-3A	0.00115	4.494	4.49515

The radioactivity was determined by the results of LSC. Four LSC counting solutions (9228-2A-1 and 9228-2A-2, 9228-3A-1 and 9228-3A-2) were counted using Packard 2500 TR LSC. A verified balance was used to weigh the actual mass of the test article solutions placed in the LSC vials. The actual sample weights and radioactivity counting results are shown in Table 3.

The radioactivity for 9228-2A and 9228-3A were determined using the following equation.

$$\text{Radioactivity (mCi)} = \frac{\text{Average LSC Counting(dpm)} \times \text{Total Weight of Diluted Test Article (g)}}{\text{Average Weight of Diluted Test Article used for LSC (g)} \times 2.22 \times 10^9 \text{ (dpm/mCi)}}$$

Example of Calculation:

$$\text{Radioactivity for 9228-3A} = \frac{363263.5 \text{ dpm} \times 4.49515 \text{ g}}{0.008 \text{ g} \times 2.22 \times 10^9 \text{ dpm/mCi}} = 0.09194 \text{ mCi}$$

Table 3. Weight of Test Article Solution Used for LSC and Results of LSC

Sample ID	Weight of 9228-2A and 9228-3A Used (g)	Average Weight of 9228-2A and 9228-3A Used (g)	LSC Counting (dpm)	Average LSC Counting (dpm)
9228-2A-1	0.008		363,564	
9228-2A-2	0.008	0.008	365,771	364667.5
9228-3A-1	0.008		367,648	
9228-3A-2	0.008	0.008	358,879	363263.5

The specific activity for the test article samples 9228-2 and 9228-3 were calculated by dividing the each total radioactivity by the each weight of test article (in grams) determined gravimetrically (using the below equation). The results of radioactivity for the test article solution and specific activity for the test article samples are shown in Table 4.

$$\text{Specific Activity (mCi/g)} = \frac{\text{Total Radioactivity (mCi)}}{\text{Weight of Test Article (g)}}$$

Example of Calculation:

$$\text{Specific Activity for 9228-3} = \frac{0.09194 \text{ mCi}}{0.00115 \text{ g}} = 79.95 \text{ mCi/g}$$

Table 4. Result of Radioactivity and Specific Activity

Sample ID	Radioactivity (mCi)[a]	Weight of Test Article Sample (g)	Specific Activity (mCi/g)[a]	Specific Activity (mCi/mmol)[b]
9228-2	0.09517	0.00074	128.6	38.07
9228-3	0.09194	0.00115	79.95	23.67

[a] Values shown in the Tables were rounded-off, calculations were performed using full precision.
[b] Molecular weight of D_4: MW =296 g/mol

The specific activity of test article 9228-2 (see Table 4) was not used for the average specific activity calculation of the two replicate. The weight of test article 9228-2 (0.00074 g) was noticed to not be in agreement with the volume of test article that the test article distributor intended to distribute. Also, there appeared to be an incorrect weight number recorded as evinced by the very reproducible radioactivity results of the replicate (see Table 4) and the specific activity determined for 9228-2 (128.6 mCi/g or 38.07 mCi/mmol) was much higher than the report value from Wizard Laboratories (69.99 mCi/g or 20.62 mCi/mmol). Therefore, the specific activity result for test article 9228-2 was not used for this study. The result of specific activity for the test article 9228-3 (79.95 mCi/g or 23.67 mCi/mmol) was recommended for the use in future studies.

3. **Radiochemical Purity**

The radiochemical purity was determined by Hewlett Packard high performance liquid chromatography (HPLC) with Packard series 500 radioactivity flow-through detector. The radiochemical purity results are summarized in Table 5. A representative HPLC radiochromatogram is shown in Figure 3.

There was no radioactivity detected in both the blank (THF) injections made onto the HPLC system. The overall average radiochemical purity for the test article, ^{14}C-D_4, was 99.563 ± 0.032 (area % ± standard deviation) based on triplicate 10 ul injections of 9228/HPLC. The radiochemical purity was evaluated and found to be within the acceptance criteria limits (>98 area %).

Table 5. Radiochemical Purity Results

Microsoft Excel Version 7.0

Study # 9228
Study Title: CHARACTERIZATION OF ^{14}C-OCTAMETHYLCYCLOTERASILOXANE (^{14}C-D_n, Lot # 990316)

Test Article: ^{14}C-OCTAMETHYLCYCLOTETRASILOXANE (^{14}C-D_4)

Replicate	Data File	Peak #	RT (min)	Peaks %	Peak Assignment
1	92280002	2	43.30	99.60	^{14}C-Octamethylcyclotetrasiloxane
2	92280003	2	43.30	99.55	^{14}C-Octamethylcyclotetrasiloxane
3	92280004	2	43.40	99.54	^{14}C-Octamethylcyclotetrasiloxane
Average Purity =		99.563			
Standard Deviation =		0.032			

Determination of Test Article Recovery

The HPLC loop check was conducted before the HPLC analysis. The collections from each injection with approximate 14 mL of scintillation cocktail were analyzed by liquid scintillation counting (Packard 2500 TR LSC). The sum of radioactivity count from the three collections for each injection was obtained (Table 6). The average radioactivity count from the three injection determination was used for calculation of the % recovery.

Table 6. Result of HPLC Loop Check for Recovery Determination

Sample ID	LSC Counting (dpm)	Sum of LSC Counting Per Injection (dpm)	Average LSC Counting (dpm)
9228/L-1-1st	366,030	366,206	365,444
9228/L-1-2nd	123		
9228/L-1-3rd	53		
9228/L-2-1st	365,000	365,196	
9228/L-2-2nd	149		
9228/L-2-3rd	47		
9228/L-3-1st	364,754	364,929	
9228/L-3-2nd	128		
9228/L-3-3rd	47		

The effluent from the third injection of the solution 9228/HPLC was collected in a tared 16 oz. glass jar. Approximate 14 ml of the effluent (9228-E) was weighed into two LSC vials (9228-E/LSC-A and 9228-E/LSC-B) and then analyzed by the liquid scintillation counter to determine the % recovery of radioactivity collected from the third HPLC tracer run. The HPLC effluent data are listed in Table 7.

Table 7. HPLC Effluent Data for Recovery Determination

Sample ID	Weight (g)	LSC Count Per Vial (dpm)	LSC Counting of Effluent[c] 9228-E (dpm)	Average LSC Counting (A&B) (dpm)
9228-E	260.63			
9227-E/LSC-A	13.96	20332	379,594	380,042
9227-E/LSC-B	14.21	20745	380,490	

$$\text{LSC Counting of Effluent (dpm)} = \frac{[\text{LSC Counting (dpm)}] \times \text{Effluent Weight in Jar (g)}}{\text{Effluent Weight in LSC Vial (g)}}$$

Example of Calculation:

$$\text{LSC Counting (dpm) from Vial A} = \frac{20332 \text{ dpm} \times 260.63 \text{ g}}{13.96 \text{ g}} = 379{,}594 \text{ dpm}$$

The effluent from the post blank injection of the solution 9228/HPLC/B was collected in a tared 16 oz. glass jar. Approximate 14 ml of the effluent (9228-EB) was weighed into two LSC vials (9228-EB/LSC-C and 9228-EB/LSC-D) and then analyzed by the liquid scintillation counter to determine the % recovery of radioactivity collected from the third HPLC tracer run. The HPLC effluent data are listed in Table 8.

Table 8. HPLC Blank Effluent Data for Recovery Determination

Sample ID	Weight (g)	LSC Count Per Vial (dpm)	LSC Counting of Effluent[c] 9228-EB (dpm)	Average LSC Counting (A&B) (dpm)
9228-EB	260.73			
9228-EB/LSC-C	13.98	78	1455	1569
9228-EB/LSC-D	14.11	91	1682	

Example of Calculation:

$$\text{LSC Counting (dpm) from Vial C} = \frac{78 \text{ dpm} \times 260.73 \text{ g}}{13.98 \text{ g}} = 1455 \text{ dpm}$$

The recovery obtained for the test article from the HPLC was calculated by the following equation. The HPLC recovery was 103.6% which was higher than 95% and it is considered to be acceptable in order for the radiochemical purity determination.

$$\% \text{ Recovery} = \frac{[\text{Average LSC Counting (dpm) (A \& B)}] - [\text{Average LSC Counting (dpm) (C \& D)}]}{\text{Average LSC Counting (dpm) (from HPLC Loop Check)}} \times 100\%$$

$$\% \text{ Recovery} = \frac{380{,}042 \text{ dpm} - 1569 \text{ dpm}}{365{,}444 \text{ dpm}} \times 100\% = 103.6\%$$

K. Conclusions

The test article, ^{14}C-octamethylcyclotetrasiloxane (^{14}C-D_4, Lot 990316), was characterized according to current EPA TSCA Good Laboratory Practice Standards. Analysis by GC/MS verified the identity of the major component of the test article, ^{14}C-D_4, as radiolabeled octamethylcyclotetrasiloxane (D_4) which met the identification as defined in the MDMS. The radiochemical purity of the test article, ^{14}C-D_4, were determined to be 99.563 ± 0.032 (area % ± standard deviation) which met the acceptance criteria for radiochemical purity by high performance liquid chromatography (HPLC) with a radioactivity flow-through detector. The mean specific activity determination of the replicate test article samples (9228-2 and 9228-3) did not meet the acceptance criteria; however, the specific activity of the test article 9228-3 (79.95 mCi/g or 23.67 mCi/mmol) was recommended for the use in future studies, as the specific activity for the test article, ^{14}C-octamethylcyclotetrasiloxane (^{14}C-D_4). The specific activity of this test article determined from this study will be used only as a guide and will be verified by study personnel prior to use in future studies.

L. Archive

The raw data was archived at the same location as the protocol, deviations, study authorization form, correspondences and final report; Dow Corning Corporation, Health and Environmental Sciences, 2200 W. Salzburg Road, Auburn, MI 48611.

M. References

1. Miller, Julie A., "Characterization of Octamethylcyclotetrasiloxane (D_4)", Dow Corning Report No.: # 1997-I0000-43659, Internal Study # 8819.
2. Miller, Julie A., "Characterization of Octamethylcyclotetrasiloxane (D_4, lot # LL098271)", Dow Corning Report No.: # 1999-I0000-46003, Internal Study # 9176.
3. Moore, J. A. in *The Analytical Chemistry of Silicones*; Smith, A. L., Ed.; Mass Spectrometry; John Wiley & Sons, Inc. New York, NY, 1991; Chapter 13.

Figure 1. Total Ion Chromatogram from GC/MS Analysis of ^{14}C-Octamethylcyclotetrasiloxane in Hexanes

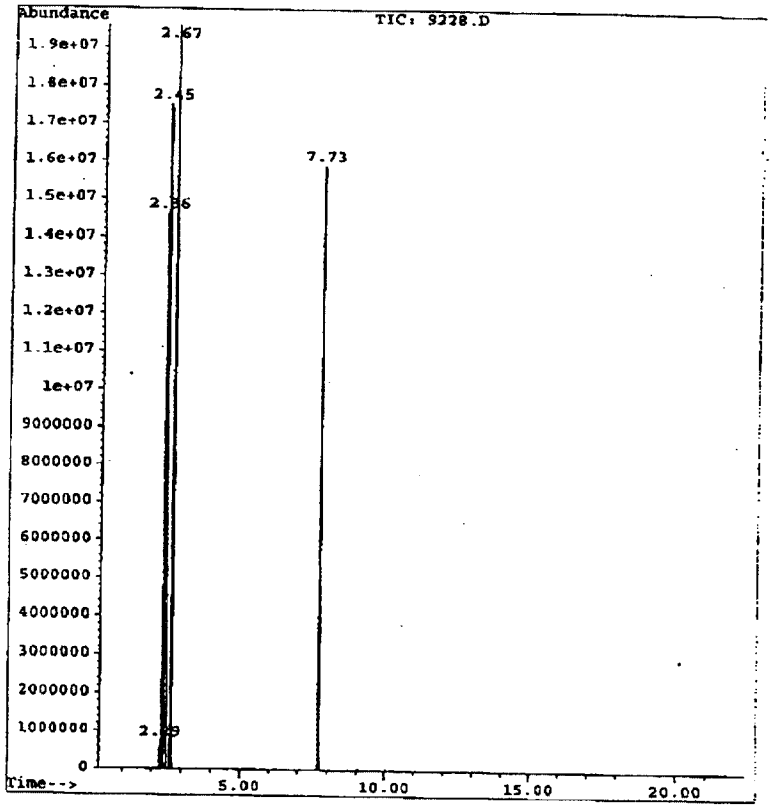


Figure 2. Mass Spectrum of ^{14}C-Octamethylcyclotetrasiloxane

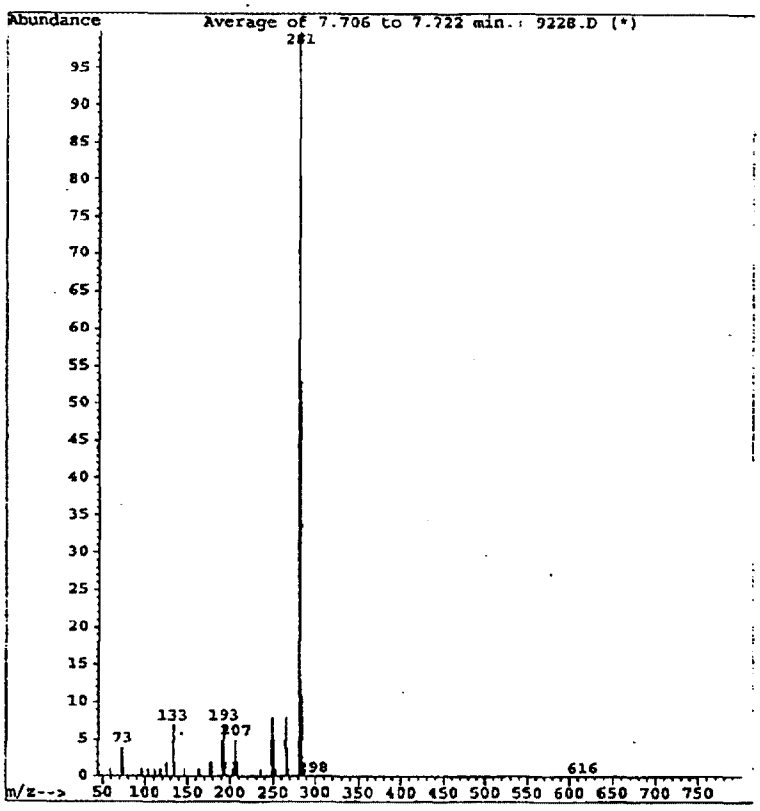
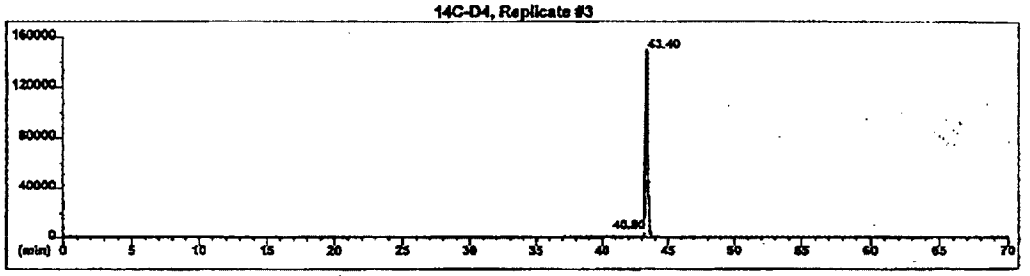


Figure 3. HPLC Radiochromatogram of ^{14}C-Octamethylcyclotetrasiloxane



Peak Report: 92280004 Channel 1 C-14 (DPM)

Pk #	Start Mins	Stop T Mins	Ret. T Mins	Net Area DPM	% Run	% Peaks
1	40.20	41.10	40.80	1608.00	0.45	0.46
2	42.90	44.30	43.40	351212.00	98.42	99.54
				352820.00	98.87	

Total Run Area: 356856

Appendix 3:Information on the Testing Facility's samples

P450 Enzyme Activities for the Pooled Sample of Human Liver Microsomes

Product No. H0610 - Lot No. 042099A

Cytochrome P450, b_5 and NADPH-cytochrome *c* reductase	**H0610 Lot No. 042099A**
Cytochrome P450 (nmol/mg)	0.395
Cytochrome b_5 (nmol/mg)	0.492
NADPH-cytochrome *c* reductase (nmol/mg/min)	244 ± 6

P450 enzyme activities (pmol/mg microsomal protein/min)		**H0610 Lot No. 042099A**
CYP1A2	7-Ethoxyresorufin O-dealkylation	49.5 ± 1.3
CYP2A6	Coumarin 7-hydroxylation	1320 ± 70
CYP2B6	*S*-Mephenytoin *N*-demethylation	156 ± 1
CYP2C8	Paclitaxel (taxol) 6α-hydroxylation	327 ± 9
CYP2C9	Diclofenac 4'-hydroxylation‡	1560 ± 10
CYP2C19	*S*-Mephenytoin 4'-hydroxylation	66.3 ± 0.7
CYP2D6	Dextromethorphan O-demethylation	317 ± 9
CYP2E1	Chlorzoxazone 6-hydroxylation	2270 ± 10
CYP3A4/5	Testosterone 6β-hydroxylation	3710 ± 140
CYP4A9/11	Lauric acid 12-hydroxylation	1600 ± 50

This pool of 15 samples contains liver microsomes from donors 16, 17, 22, 23, 24, 25, 26, 27, 31, 32, 35, 36, 38, 40 and 41. This product was prepared on 04/20/99 from identical amounts of liver microsomes from each donor. Values for enzyme activities are mean ± standard deviation of three or more determinations.

‡ Rates for the formation of 4'-hydroxydiclofenac were calculated using the response factor (AUC/pmol) for the substrate, diclofenac.

Note: Not all enzyme activity values have been quality assured.

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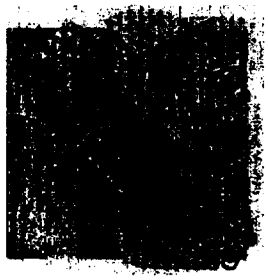
(Continued)

Appendix 3 continued

3800 Cambridge, Kansas City, KS 66103 USA
Phone: 913.588.7530 • Fax: 913.588.7572
US Toll Free: 877.588.7530 (Customer Service)
www.xenotechllc.com



R1073 / Lot No. 051499A
Sprague Dawley Rat Liver Microsomes
Saline-treated
Male, Pool of 42
0.5 mL at 10 mg protein / mL

Specific content and activities	Content / Rate
Cytochrome P450 (nmol/mg protein)	0.796
Cytochrome b_5 (nmol/mg protein)	0.387
NADPH-cytochrome *c* reductase (nmol/mg protein/min)	215

Background: Liver microsomes from male rats treated with saline are intended to serve as vehicle-treated controls for studies with liver microsomes from male rats treated with certain P450 enzyme inducers, such as phenobarbital, isoniazid, streptozotocin and clofibric acid.

Note: XenoTech also offers liver microsomes from untreated rats (R1000) and rats treated with corn oil (R1098).

Animal Information

Species:	Rat	Treatment:	Saline (0.9% NaCl irrigation solution)
Strain:	Sprague Dawley	Source:	Baxter (Cat. No. 2F7123)
Sex:	Male	Volume injected:	5 mL/kg body weight
Age:	~8 weeks	Regimen:	Once per day on days 1-4, liver microsomes prepared on day 5
Vendor:	Harlan, Indianapolis, IN		

Rats were laboratory animals and were housed in an AAALAC-accredited facility, which is registered as a research facility with the USDA-APHIS-AC. They were allowed to acclimate seven days before use.

Food:	Purina Rodent Laboratory Chow #5001 *(ad libitum)*
Water:	Automatic watering system (*ad libitum*)
Light/dark cycle:	6:00 am – 6:00 pm light, 6:00 pm – 6:00 am dark (12-hour light/dark)
Temperature:	72°F ± 3°F
Humidity:	45-55%
Bedding:	Cell-Sorb Plus (gypsum treated paper product), A&W Products, New Philadelphia, OH
Cage:	Polycarbonate Shoebox Cage, conventional cage

Store at -80°C

CAUTION: Although strict measures are taken to ensure that livers obtained from laboratory animals do not harbor infectious diseases, we recommend that all animal products be handled as potential biohazards and universal precautions be followed.

For in vitro use only

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www.xenotechllc.com

R1078 / Lot No. 070297A
Sprague Dawley Rat Liver Microsomes
Phenobarbital-treated
Male, Pool of 21
0.5 mL at 10 mg protein / mL

Specific content and activities	Content / Rate
Cytochrome P450 (nmol/mg protein)	2.31
Cytochrome b_5 (nmol/mg protein)	0.689
NADPH-cytochrome *c* reductase (nmol/mg protein/min)	448
7-pentoxyresorufin *O*-dealkylation (pmol/mg protein/min)	1490 [a]

[a] Fold induction: ~85-fold increase over control microsomes

Background: Treatment of rats with the peroxisome proliferator, phenobarbital, causes a marked induction (>10-fold) of liver microsomal CYP2B levels, which is associated with an increase in 7-pentoxyresorufin, *O*-dealkylation 7-benzyloxyresorufin *O*-dealkylation and testosterone 16β-hydroxylation. Liver microsomes from saline-treated rats (Cat. No. R1073) were used as a control. The results confirm the anticipated induction of CYP2B activity.

Animal Information

Species:	Rat	Treatment:	Phenobarbital
Strain:	Sprague Dawley	Source:	Spectrum Chemical Co. (Cat. No. 4119)
Sex:	Male	Vehicle:	Saline
Age:	~8 weeks	Concentration:	16 mg/mL
Vendor:	Harlan, Indianapolis, IN	Regimen:	80 mg/kg body weight once per day on days 1-4, liver microsomes prepared on day 5

Rats were laboratory animals and were housed in an AAALAC-accredited facility, which is registered as a research facility with the USDA-APHIS-AC. They were allowed to acclimate seven days before use.

Food:	Purina Rodent Laboratory Chow #5001 *(ad libitum)*
Water:	Automatic watering system (*ad libitum*)
Light/dark cycle:	6:00 am – 6:00 pm light, 6:00 pm – 6:00 am dark (12-hour light/dark)
Temperature:	72°F ± 3°F
Humidity:	45-55%
Bedding:	Cell-Sorb Plus (gypsum treated paper product), A&W Products, New Philadelphia, OH
Cage:	Polycarbonate Shoebox Cage, conventional cage

Store at -80°C

CAUTION: Although strict measures are taken to ensure that livers obtained from laboratory animals do not harbor infectious diseases, we recommend that all animal products be handled as potential biohazards and universal precautions be followed.

For in vitro *use only*

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Appendix 4: Method validation for the HPLC analysis of Octamethycyclotetrasiloxane (D_4) and its metabolites in *in vitro* incubations with human liver microsomes

Abstract

The purpose of experiments in this section was to validate a high performance liquid chromatography (HPLC) method to be used for the determination of concentrations of D_4 (substrate) and its metabolite(s) in samples obtained from an *in vitro* metabolism study of D_4. The method utilized reversed-phase chromatography with radiometric detection.

The lower limit of quantification (LLQ) was determined to be 0.4 µM (40 pmol on column) for D_4. The radiometric detector response was linear (r^2 = 0.991) up to the highest concentration examined (4 µM). Since the concentration of the test article (D_4) in the study was designed to be 3 µM, the highest concentration for the calibration curves was set at 4 µM, 33% above the substrate concentration of the study. The upper limit of quantification (ULOQ) was not determined.

Calibration curves were generated from the radiometric detector response for D_4 in the range of 0.4 to 4 µM (40 to 400 pmol injections). Calibration curves consisted of six concentrations, plus blank, in duplicate. Accuracy ranged from 92% to 112%. Between-day precision (coefficient of variation) of back-calculated concentrations for the calibration curves were all less than 10%.

Concentrations from within the calibration curves were also used to assess freeze/thaw stability. The analysis of three concentrations (0.4, 1.6, and 4 µM, in triplicate analysis) demonstrated that the samples were stable through at least three freeze/thaw cycles. This was examined by injecting the three concentrations mentioned above after they were frozen at –80 ±5°C for 1, 2 and 3 cycles.

In-process stability was demonstrated by injecting three concentrations (0.4, 1.6, and 4 µM, in triplicate analysis) after the samples were kept at room temperature for approximately 24 hours. The absolute mean difference from the nominal amount were within 4% which suggested that the samples were stable through at least 24 hours at room temperature.

Introduction

This study was designed to determine the role of human liver microsomal enzymes in the *in vitro* metabolism of D_4. Part 1 of the protocol was designed to develop and validate an HPLC procedure to resolve and quantify D_4 and its major metabolites.

Materials and methods

Materials

Chemicals

ß-NADPH, sucrose, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). EDTA and THF were purchased from Aldrich (Milwaukee, WI). Potassium phosphate was purchased from J.T. Baker (Phillipsburg, NJ). All solvents were of HPLC grade. Water obtained from the Millipore® water purification system was used to prepare aqueous solutions.

Test Article: D_4

D_4 was received by the Testing Facility from Wizard Laboratories, Inc. (West Sacramento, CA) on July 9, 1999 together with a Certificate of Analysis (**Appendix 1**). In addition, a characterization report (report No. 1999-I0000-47184) on the test article was provided by the Sponsor (**Appendix 2**).

Quantity received:	1.0 mCi
Specific activity:	20.62 Ci/mol
Molecular weight:	296.62g/mol
Lot number:	990316
Solvent used for dissolution:	Anhydrous ethanol
Clarity in anhydrous ethanol:	Clear
Purity:	99.67%
Storage of stock solution:	Anhydrous ethanolic solution was stored in crimp capped clear plastic vials at –80 ±5°C.

Source of liver microsomes

Human liver microsomes (pool of 15 individuals, Cat No. H0610, Lot No. 042099A) were used for this study. These microsomes were prepared and characterized at the Testing Facility with respect to the activities of various P450 enzymes (**Appendix 3**).

Methods

To study the *in vitro* metabolism of D_4, an HPLC procedure was developed to separate D_4 from its metabolite(s). This procedure was based on an HPLC method provided by the Sponsor. The HPLC method used in this study is described below.

Chromatographic conditions

1. HPLC System: Shimadzu, Model LC-10A
2. Radiometric Detector: IN/US Flow-Through System, β-RAM Model 2
3. Analytical Column: Alltech, (Alltima C-18, Part No. 88056, Column No. 99050599, Lot No. 1062, 4.6 mm x 250 mm, 5 µm particle)
4. Guard Column: Alltech, (Alltima C-18, Part No. 96080, 5 µm particle)
5. Mobile phase: (A) Millipore Water, (B) Acetonitrile, Binary Gradient
6. Flow rate: 1.0 mL/min
7. Injection volume: 200 µL
8. HPLC run time: 70 minutes
9. Gradient profile

Time (min)	% mobile phase B	Gradient profile
0-20	0	Hold
20-40	0 to 100	Linear gradient
40-50	100	Hold
50-55	100 to 0	Linear gradient
55-70	0	Re-equilibrate

Assessment of the linearity of the detector response and determination of the lower limits of quantification (LLQ)

Zero-time incubation samples were prepared to assess the linear range of the radiometric detector response for D_4. Linearity and the LLQ were determined in the presence of biological matrix (microsomal incubation mixture) by comparing the radiometric detector response (peak area-under-the-curve) with the amount of D_4 injected on the HPLC column. HPLC analysis of the standards (40, 80, 120, 160, 200, 240, 280, 320, 360 and 400 pmol D_4) were performed in duplicate. These samples were prepared by adding substrate (D_4) to a solution containing buffer mixture and protein. Briefly, zero-time incubations (final volume 0.35 mL) containing human liver microsomes (pool of 15, 1 mg protein/mL), $MgCl_2$ (3 mM), potassium phosphate buffer (50 mM, pH 7.4), EDTA (1 mM, pH 7.4), and D_4 (0.4-4.0 µM), at the final concentrations indicated, were mixed with 0.4 mL of THF and then 0.05 mL of ß-NADPH (50-250 µM) was added. A stock solution of D_4 was prepared in anhydrous ethanol (top concentration of 9.7 mM). This stock solution was diluted to achieve the final concentrations described above, and the final concentration of anhydrous ethanol was 0.5% in all samples before the addition of the stop reagent (0.4 mL THF). The precipitated protein was removed by centrifugation (920 x *g* for 10 min at 10°C), and an aliquot (200 µL) of the supernatant fractions were analyzed by HPLC with the method described above. Regression coefficient (r^2) was calculated to assess the linearity of the detector. The LLQ for D_4 was determined according to the precision and accuracy based on the criteria described below.

Validation of the calibration curves

A three-run validation of calibration curves was performed. HPLC analyses of the standard mixtures (range of 0.4-4 µM; 40, 80, 160, 240, 320, 400 pmol) were performed in duplicate and validated based on its linearity, differences of the back-calculated standards from the nominal amounts of the analytes (percent deviation from nominal) and percent accuracy. Between-day precision (coefficient of variation) of back-calculated concentrations for the calibration curves were determined. These samples were prepared by mixing mock incubation mixtures with known amounts of D_4 as described in the previous section. The calibration curves were freshly prepared from working solutions for each validation run.

Acceptance criteria

Correlation coefficients (r) must be ≥ 0.980 for the standard curves. The differences of the back-calculated standards from the nominal amounts or concentrations of the analytes should be $\leq 20\%$. For a single qualification run (in duplicate), a maximum of two standard analyses per 10 standard analyses may be excluded from the standard curve if its back-calculated value does not meet these criteria. The excluded point may be the highest or lowest concentration in the standard curve, provided both replicates are not excluded.

Accuracy and Precision

Due to the nature of D_4 (high volatility), QC samples were not prepared. As a result, the precision of the method was determined by determining the relative standard deviations (RSDs) for the three calibration curves as between-day precision. The accuracy of the method was determined by triplicate analyses of three concentrations consisting of the highest, middle and lowest (4, 1.6, 0.4 µM, respectively) levels used for the calibration curve. These samples for the accuracy test were prepared by the same procedure as described above. Three replicates from each of the mentioned concentrations were analyzed on Method Validation-2 Repeat. Differences of the back-calculated amount from the nominal amount of D_4 (percent deviation from nominal) and percent accuracy were calculated.

Acceptance criteria

Accuracy

For each run, the mean back-calculated concentrations for the samples were to be between 80% and 120% from the nominal values.

Precision

The relative standard deviations (RSDs) for the back-calculated concentrations for the calibration samples were to be $\leq 20\%$ from the nominal values.

Stability of the samples

Reproducibility and stability of the calibration curve was examined by injection of three concentrations (0.4, 1.6 and 4 µM) that had been frozen for 3, 4, and 5 days at -80±5°C in three cycles. These concentrations were prepared from the same substrate solutions and on the same day as the fresh (Day 1) samples. All stability samples were thawed at room temperature on Day 3 and Cycle 1 samples were analyzed by HPLC. Cycle 2 and 3 samples were refrozen at -80±5°C. All remaining frozen stability samples were thawed to room temperature on Day 4 and Cycle 2 samples were analyzed by HPLC. Cycle 3 samples were refrozen at -80±5°C and then thawed to room temperature on Day 5 and analyzed by HPLC. Differences of the back-calculated amount from the nominal amount of D_4 (percent deviation from nominal) and percent accuracy were calculated using the slope and intercept generated by the Day 1 calibration curve. In addition, three concentrations of standards (0.4, 1.6 and 4 µM) stored at room temperature for approximately 24 hours were analyzed in triplicate to assess in-process stability of the samples.

Acceptance criteria

The mean back-calculated concentration of the samples under each storage condition must have a mean difference ≤ 20% from the values from the nominal amounts of D_4 in the samples.

Data processing

All raw data (chromatographic peak areas) were manually entered to a spreadsheet computer program, Microsoft Excel (Microsoft Corp., Seattle, WA). The calibration line parameters were determined by transferring all the data electronically from Excel to a computer software package, GraFit (Version 4.06, Erithacus Software Limited, London, UK) using linear regression analysis with statistical-weighting. The amount of analytes detected in samples were then back-calculated using the appropriate calibration curve.

Records

Lot numbers of the standards, solution I.D., solution preparation worksheets (*e.g.*, standard stock, calibration standards, mobile phase) and detailed experimental procedures were stored in the Study book XT052398, Method Validation Section.

Results

Representative HPLC chromatograms obtained from incubation of D_4 with human liver microsomes are shown in **Appendix 4-Figure 1**. The metabolite was adequately resolved from D_4. The retention times for D_4 and its metabolite were approximately 50 and 45 minutes, respectively.

Assessment of the linearity of the detector response and determination of the lower limits of quantification (LLQ)

A linearity experiment established that a reliable LLQ was 0.4 μM for D_4 (**Appendix 4-Table 1**). The radiometric detector response was linear (r^2 = 0.991) up to the highest concentration examined (4 μM, **Appendix 4-Figure 2**). Since the concentration of the test article (D_4) in the study was designed to be 3 μM, the highest concentration for the calibration curves was set at 4 μM, 33% above the substrate concentration of the study.

Validation of the calibration curves

Calibration curves were generated from the radiometric detector response for D_4 in the range of 0.4 to 4 μM (six points, 40 to 400 pmol per injection) plus blank (however, the blank point was not used to construct calibration curves) in duplicate. **Appendix 4-Tables 2a** through **2c** summarize the results of Day 1 through Day 3, respectively, calibration curves for the analyte. Percent deviation from nominal of the back-calculated analyte (pmol per injection) and percent accuracy were calculated for each concentration. Accuracy ranged from 92% to 112%.

Accuracy and Precision

Between-day precision (coefficient of variation) of back-calculated concentrations for the calibration curves were all less than 10% (**Appendix 4-Table 3**). Three concentrations (0.4, 1.6 and 4 μM) of samples were prepared and analyzed in triplicate on Method Validation Day-2 Repeat. Differences of the back-calculated amount from the nominal amount of D_4 (percent deviation from nominal) and percent accuracy were calculated. Accuracy ranged from 101% to 104% (**Appendix 4-Table 4**).

Stability of the samples

Stability of the samples at room temperature and after freeze/thaw cycles were examined and the results are shown in **Appendix 4-Table 5**. Differences of the back-calculated amount from the nominal amount of D_4 (percent deviation from nominal) and percent accuracy were calculated. Percent accuracy for room temperature stability samples ranged from 89% to 111%. The absolute mean difference from the nominal values were within 4% which suggested that the samples were stable through at least 24 hours at room temperature. Percent accuracy for the freeze/thaw samples ranged from 98% to 119% with an exception of 0.4 μM freeze/thaw Cycle 1 sample (138%, cause remains unknown).

Conclusions

An HPLC method has been validated for the determination of D_4 (Octamethylcyclotetrasiloxane) and its metabolite in the range of 0.4 to 4 μM in *in vitro* incubations with human liver microsomes. The reproducibility of the calibration curves were excellent, and the in-process stability indicated that samples could remain stable on the autosampler (at room temperature)

for at least 24 hours. Also, samples can be thawed and refrozen at least three times, if necessary, without losing sample integrity.

List of Tables

List of Figures

Table 1: Linearity assessment of the radiometric-detector response for the detection of D_4

Concentration (µM)	Analyte per injection (pmol)	Back-calculated pmol[a]	Percent deviation from nominal	Percent Accuracy	Mean back calculated pmol[b]
4 µM	400	416	3.9	103.9	418
		420	5.1	105.1	
3.6 µM	360	355	-1.4	98.6	355
		ND[c]	ND	ND	
3.2 µM	320	336	5.1	105.1	325
		313	-2.1	97.9	
2.8 µM	280	275	-1.7	98.3	265
		255	-9.0	91.0	
2.4 µM	240	251	4.4	104.4	253
		255	6.4	106.4	
2 µM	200	198	-0.9	99.1	198
		ND[d]	ND	ND	
1.6 µM	160	146	-8.5	91.5	145
		144	-10.1	89.9	
1.2 µM	120	111	-7.3	92.7	119
		126	5.1	105.1	
0.8 µM	80	87.8	9.7	109.7	83.2
		78.7	-1.6	98.4	
0.4 µM	40	43.0	7.6	107.6	43.0
		ND[e]	ND	ND	

Calculated parameters for the linearity of the detector response

Intercept:	-15077.03012
Gradient:	1375.63282
Correlation coefficient (r):	0.99549
Coefficient of determination (r^2):	0.99100

[a]: (Peak AUC-intercept)/gradient (values are rounded to three significant figures)

[b]: Mean of two determinations (values are rounded to three significant figures)

ND[c]: Not Determined due to misinjection, ND[d]: Not determined due to >20% deviation from nominal, ND[e]: no peak detected

Percent Accuracy = (Back-calculated pmol/Analyte per injection) x 100

Table 2a: Summary of the calibration curves-Day 1

Concentration (μM)	Analyte/injection (pmol)	Mean back-calculated analyte/injection (pmol)[a]	Mean % Accuracy[b]
4 μM	400	404	101.1
3.2 μM	320	314	98.1
2.4 μM	240	233	97.3
1.6 μM	160	169	105.7
0.8 μM	80	83.4	104.2
0.4 μM	40	38.6	96.6

[a]: Mean of two determinations. All values are rounded to three significant figures
[b]: Calculated with Excel using the values that were not rounded. Results were rounded to one decimal place.
Performed on 10/11/99

Table 2b: Summary of the calibration curves-Day 2

Concentration (μM)	Analyte/injection (pmol)	Mean back-calculated analyte/injection (pmol)[a]	Mean % Accuracy[b]
4 μM	400	428	107.1
3.2 μM	320	303	94.6
2.4 μM	240	227	94.5
1.6 μM	160	171	107.0
0.8 μM	80	75.8	94.8
0.4 μM	40	44.7	111.7

[a]: Mean of two determinations. All values are rounded to three significant figures
[b]: Calculated with Excel using the values that were not rounded. Results were rounded to one decimal place.
Performed on 11/1/99

Table 2c: Summary of the calibration curves-Day 3

Concentration (μM)	Analyte/injection (pmol)	Mean back-calculated analyte/injection (pmol)[a]	Mean % Accuracy[b]
4 μM	400	406	101.5
3.2 μM	320	299	93.4
2.4 μM	240	252	104.8
1.6 μM	160	166	103.6
0.8 μM	80	87.9	109.8
0.4 μM	40	36.8	91.9

[a]: Mean of two determinations. All values are rounded to three significant figures
[b]: Calculated with Excel using the values that were not rounded. Results were rounded to one decimal place.
Performed on 11/16/99

Table 3: Summary of the calibration curves: Between-day precision

Concentration (μM)	Day 1[a]	Day 2[b]	Day 3[c]	Mean[d]	S.D.	%CV
4 μM	404	428	406	413	13.4	3.24
3.2 μM	314	303	299	305	7.75	2.54
2.4 μM	233	227	252	237	12.8	5.40
1.6 μM	169	171	166	169	2.80	1.66
0.8 μM	83.4	75.8	87.9	82.4	6.08	7.38
0.4 μM	38.6	44.7	36.8	40.0	4.14	10.3

[a]: Mean back-calculated analyte/injection (pmol) for Day 1
[b]: Mean back-calculated analyte/injection (pmol) for Day 2
[c]: Mean back-calculated analyte/injection (pmol) for Day 3
[d]: Mean of the data from Day 1,2 and 3

Calibration curves y = mx + b (y: peak area, x: pmol of analyte, m: gradient, b: intercept)

Assay	m (slope)	b (intercept)	r^2
Day 1	1435.7505	-13591.3605	0.9960
Day 2	1517.0307	-7063.7551	0.9853
Day 3	1350.1623	-12613.3449	0.9932

All values are rounded to four decimal points

Table 4: Summary of the accuracy of the method

Concentration (μM)	Analyte/injection (pmol)[a]	Mean back-calculated analyte/injection[b]	S.D.	%CV	Mean % Accuracy
4 μM	400	403	7.6	1.9	100.8
1.6 μM	160	167	6.3	3.8	104.1
0.4 μM	40	41.4	4.9	11.8	103.4

[a]: Theoretical amount of D_4 injected on column
[b]: Mean of three determinations. Values are rounded to three significant figures
Performed on 11/1/99

Table 5: Summary of the stability assessment (in-process and freeze/thaw cycles)

D_4 (µM)	Nominal amount/injection (pmol)	Sample[a]	Back-calculated analyte/injection (pmol)	Percent accuracy[b]	Mean back-calculated analyte/injection (pmol)	Mean % Accuracy
4 µM	400	F/T 1 cycle	420	105.0	405	101.1
		F/T 2 cycles	400	100.0		
		F/T 3 cycles	394	98.4		
1.6 µM	160	F/T 1 cycle	175	109.1	176	110.0
		F/T 2 cycles	177	110.8		
		F/T 3 cycles	ND	ND		
0.4 µM	40	F/T 1 cycle	55.2	138.1	49.4	123.5
		F/T 2 cycles	45.2	113.0		
		F/T 3 cycles	47.8	119.4		
4 µM	400	RT for 18 hrs	400	99.9	403	100.8
			412	103.0		
			398	99.5		
1.6 µM	160	RT for 24 hrs	163	101.7	167	104.1
			163	102.1		
			174	108.7		
0.4 µM	40	RT for 27 hrs	44.5	111.2	41.4	103.4
			43.8	109.6		
			35.7	89.4		

[a]: F/T (Freeze/Thaw) cycles: Single determination/cycle/concentration. RT (room temperature): Hours shown are the time since the samples were placed on HPLC loading rack until injection time.

[b]: Percent accuracy = (Back-calculated analyte per injection)/(Nominal amount per injection) x 100

ND: Not determined

Figure 1: Representative HPLC chromatograms of incubations obtained from D_4 (3 μM) with human liver microsomes

[^{14}C]-D_4 (3 μM, 49.5 nCi/incubation) was incubated with human liver microsomes (pool of 15, 1 mg protein/mL) in a buffer mixture containing potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM, pH 7.4), and β-NADPH (187.5 μM) at the final concentrations indicated (final incubation volume 800 μL). Incubation was carried out at 37±1°C in 1-mL size crimp capped plastic vial for 60 min. Reaction was initiated by adding β-NADPH using a plastic syringe (B-D, 1 mL syringe with a 23-gauge stainless steel needle) and was terminated by transferring one half of the final incubation volume (400 μL) to a crimp capped glass HPLC vial containing an equal volume of THF (stop reagent). Tube was centrifuged at 920 x *g* for 10 min at 10°C to precipitate protein. Following centrifugation, an aliquot (200 μL) of the supernatant fraction was analyzed by HPLC (radiometric detection).

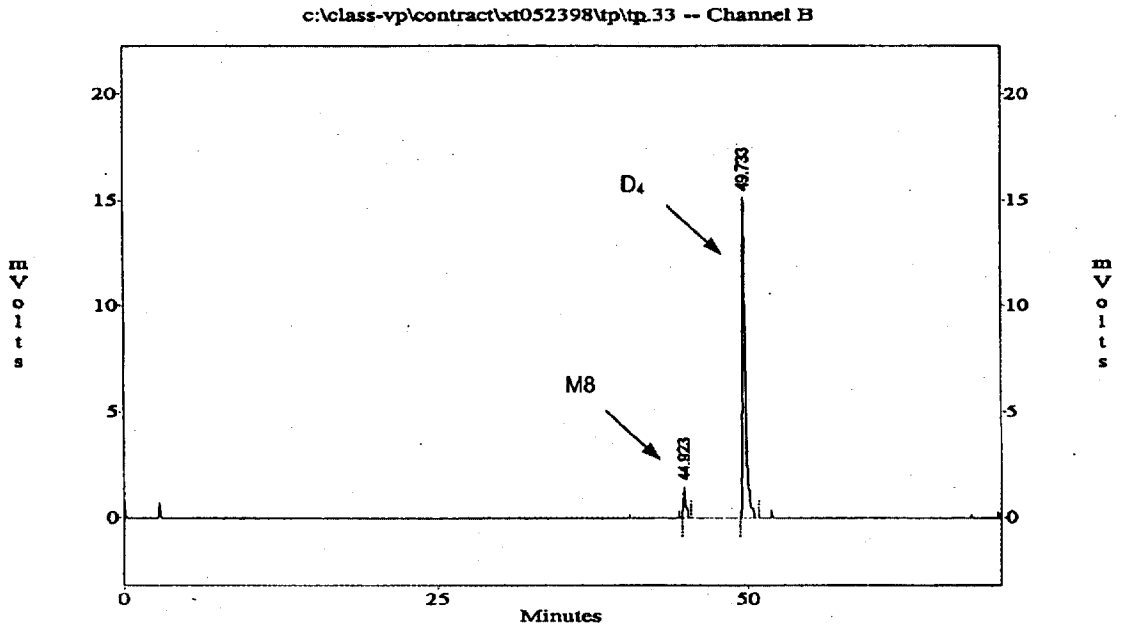
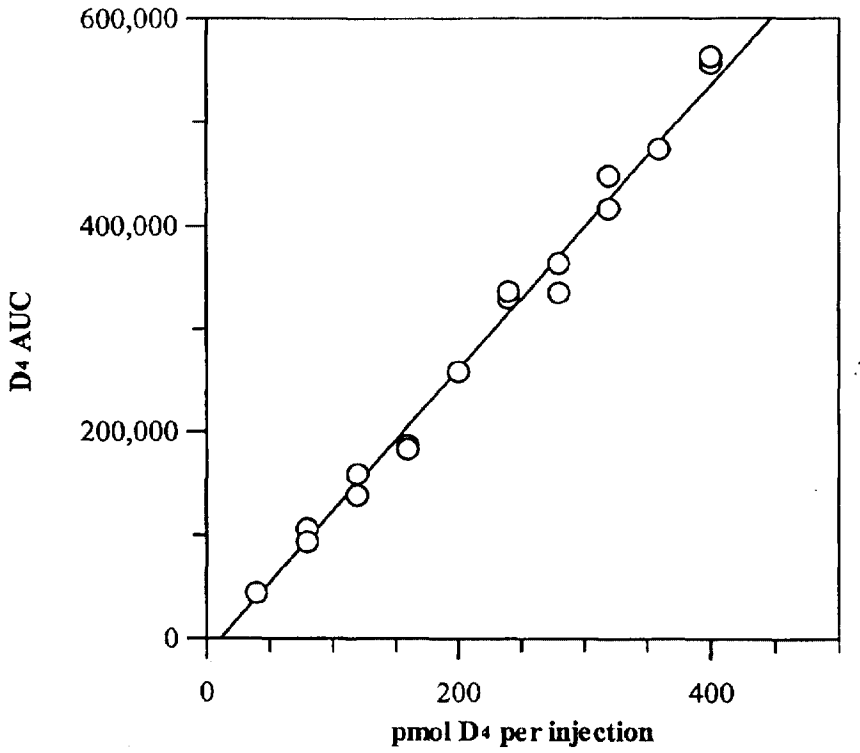


Figure 2: Linearity assessment of the radiometric detector response for the detection of D_4



Parameter	Value	Std. Error
a (intercept)	-15077.03012	5978.76072
b (gradient)	1375.63282	33.84098

Appendix 5: Copy of the protocol and amendments

XenoTech Protocol XT 052398
(Dow Corning Study No. 8956)

***In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes**

Testing Facility
XenoTech, LLC
3800 Cambridge
Kansas City, KS 66103

Sponsor
Dow Corning Corporation
2200 W. Salzburg Rd.
Midland, MI 48686-0994

Date Printed: June 1, 1999

Protocol Distribution List
Study Director
Study Manager
Quality Assurance Unit
Participating Analysts
Office Manager
Sponsor Representative/Monitor

I. STUDY PURPOSE, OVERALL DESIGN and BACKGROUND

This protocol is designed to determine the role of human liver microsomal enzymes in the *in vitro* metabolism of D_4. Part 1 of this protocol is designed to develop and validate an HPLC procedure to resolve and quantify D_4 and its major metabolites. Part 1 of this protocol will also determine if D_4 is metabolized by liver microsomes from humans or other laboratory animals. Part 2 of this protocol is designed to characterize the metabolism of D_4 by human liver P450 enzymes and to identify which specific P450 enzyme or enzymes are responsible for metabolizing D_4.

II. PERSONNEL INVOLVED IN THE STUDY

Sponsor/Study Monitor
Kathy Plotzke, Ph.D.
Toxicology Research Manager
Dow Corning Corporation
2200, W. Salzburg Rd.
Midland, MI 48686-0994

Tel. (517) 496 8046
Fax (517) 496 5595

Testing Facility
XenoTech LLC
3800 Cambridge
Kansas City, KS 66103

Study Director
Etsuko Usuki, Ph.D., Senior Scientist

Tel. (913) 588 7495
Fax (913) 588 7572

Study Manager
Ajay Madan, Ph.D., Principal Scientist

Tel. (913) 588 7509
Fax (913) 588 7572

Quality Assurance Unit
Kelly Bunce, B.S.
XenoTech LLC

Office Manager
Patti St. Vincent
XenoTech LLC

Proposed Participating Analysts
Brian Ogilvie, B.S. Scientist

III. PROPOSED TEST SCHEDULE

Proposed experimental start date:	To be determined
Proposed experimental termination date:	To be determined
Target date for submission of draft unsigned report:	To be determined
Target date for submission of final report:	To be determined

IV. GLP COMPLIANCE STATEMENT

This study will be conducted in accordance with the Environmental Protection Agency (EPA) Good Laboratory Practice (GLP) Regulations, 40 CFR Part 792.

V. TEST SYSTEM

Human liver microsomes from individual livers or a pool (pooled from five or more individuals) will be used for this study. These human liver microsomes have been characterized by the Testing Facility with respect to P450 enzyme activities.

VI. TEST ARTICLE SPECIFICATION:

D_4-unlabeled:

Identification:	D_4
Lot Number:	LL084732
CAS Number:	556-67-2
Physical Description:	Liquid
Storage Conditions:	Ambient
Handling Precautions:	As Described in the MSDS provided by the Sponsor
Stability:	30 months
Purity:	99.8%
Analytical Procedure to Verify Test Article:	GC/MS
Reserve Sample:	Sponsor will hold sample in reserve
Solvent for Preparation of Solution:	Anhydrous Ethanol
Exposure Concentrations to be Tested:	Determined during the study and will be documented in the final report
Archive Requirement:	To be archived by Sponsor

[^{14}C]-labeled D_4:

Identification:	[^{14}C]-D_4
Lot Number:	To be determined
CAS Number:	N/A
Specific Activity:	To be documented in the study file and the final report
Physical Description:	Clear liquid
Storage Conditions:	-80°C
Handling Precautions:	As Described in the MSDS provided by the Sponsor
Stability:	30 months
Purity:	To be documented in the study file and the final report
Analytical Procedure to Verify Test Article:	HPLC/Radiomatic flow detector and GC/MS
Reserve Sample:	Sponsor will hold sample in reserve
Solvent for Preparation of Solution:	Anhydrous Ethanol
Concentrations of Substocks:	To be determined
Exposure Concentrations to be Tested:	Determined during the study and will be documented in the final report
Archive Requirement:	To be archived by Sponsor

VII. EXPERIMENTAL DESIGN AND PROCEDURES

Test System (Rationale and characterization):

The major P450 enzymes in human liver microsomes are CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4A9 and CYP4A11. There is enormous sample-to-sample variation in the expression of these P450 enzymes in individual human liver microsomal samples. Therefore, to minimize this variation, pooled microsomes from several human livers will be used for most experiments. In addition, microsomes from individual livers will be used to ascertain sample-to-sample variation in the metabolism of D_4. All liver microsomes were prepared and characterized by the Testing Facility (Pearce *et al.*, 1996) according to SOP XT 03, XT 07, XT 08, XT 09, XT 10, XT 12, XT 15, XT 16, XT 18, XT 21, XT 23, XT 25, XT 40 and/or XT 48.

Test article

Selection of exposure concentrations:

The choice of substrate concentration (D_4 concentration) is very important if meaningful kinetic constants are to be determined. Ideally, the choice of D_4 concentration is determined by the observed maximal concentration (Cmax) of the drug in the plasma or liver of either animals or humans. However, if this information is not available or if the sensitivity of the analysis does not permit evaluation of submicromolar concentrations of D_4, experiments may be carried out at concentrations as high as 3.0 μM. This concentration is at least 40 fold higher than its limit of aqueous solubility (50 ppb). A concentration higher than the aqueous solubility can be studied because D_4 is solubilized by microsomal protein present in the incubation mixtures. The concentrations of D_4 used for reaction phenotyping will be based on the results of Parts 1 and 2 as discussed below (in Part 2 of the Experimental Design). Incubations containing no D_4 will contain the organic solvent used to dissolve D_4 (*e.g.*, anhydrous ethanol).

Handling and Disposal:

[^{14}C]-labeled D_4 will be provided by the Sponsor and shipped to the Testing Facility, where it will be stored as specified. Test article characterization and radio-chemical purity will be determined by the Sponsor and provided to the Testing Facility. In addition, the Testing Facility will verify the radio-chemical purity by the HPLC method provided by the Sponsor. The concentration of D_4 in the working solutions prepared fresh for each experiment will be determined based on the specific activity of D_4 stock used. A log-sheet will be maintained to document usage and fate of the test article. At the end of the study any unused D_4 will be shipped back to the Sponsor or disposed in a manner specified by the Sponsor.

PART 1. Evaluation of the metabolism of D_4 by human liver microsomes

The following experiments assume that a detailed HPLC method for analysis of D_4 will be provided by the Sponsor. In addition, any information on the metabolism of D_4 should be provided to the Testing Facility. The method validation data on the HPLC analysis will be documented in the raw data and summarized in the final report. The experiments outlined in Part 1 will be carried out with microsomes (pooled from five or more human livers) which have been characterized previously.

Experiment 1a. HPLC set-up and evaluation of suitability of the HPLC method

To study the *in vitro* metabolism of D_4, an HPLC procedure will be established to separate D_4 from its metabolites. This procedure will be based on an HPLC method provided by the Sponsor. D_4 and its metabolites will be detected by a procedure provided by the Sponsor. If necessary, the HPLC procedure provided by the Sponsor may be modified to separate D_4 from its metabolites. Therefore, preliminary experiments will be carried out to evaluate the suitability of the HPLC method before initiating HPLC method validation. The actual conditions for this experiment are described in Experiment 1b.

Experiment 1b: *In vitro* metabolism of D_4 by human liver microsomes

D_4 will be incubated with microsomes from human livers. The formation of metabolites of D_4 and the disappearance of the parent compound will be studied in the absence or presence of NADPH. If the formation of metabolites were dependent on NADPH then cytochrome P450 and/or FMO would be implicated in the metabolism of D_4.

Liver microsomes (0.1 to 2 mg/ml) will be incubated at 37±1°C in incubation mixtures (final volume 0.1 to 2 ml) containing potassium phosphate buffer (25-100 mM, pH 7.4±0.1), $MgCl_2$ (3 mM), EDTA (1 mM) and D_4 (*e.g.*, 0.1, 1.0 and 3 µM) with and without β-NADPH. D_4 will be added to each incubation in ethanol (final concentration of ethanol will not exceed 0.5%). Reactions will be started by addition of β-NADPH (final concentration 50-250 µM) or by the addition of D_4, and will be stopped after 60 min by the addition of a stop reagent (*e.g.*, tetrahydrofuran). Zero-time, zero-protein and zero-substrate incubations will serve as blanks. Precipitated protein will be removed by centrifugation (400-2,500 *g* for 5-15 min at 5-15°C). An aliquot (up to 500 µl) of the supernatant fraction will be analyzed by HPLC as described in Experiment 1a.

If incubating as much as 3 µM D_4 with 2 mg of liver microsomal protein for 60 min in the presence of β-NADPH results in no detectable formation of metabolites, and if incubating as little as 0.1 µM D_4 with 2 mg of liver microsomal protein for 60 min in the presence of β-NADPH results in no detectable loss of parent compound, it will be assumed that D_4 is minimally metabolized by cytochrome P450 and/or FMO. The loss of parent compound will be assessed by comparing the radiometric detector response (peak area-

under-the-curve, peak AUC) with the known amount of D_4 in the presence of the biological matrix (zero-time microsomal incubation mixtures).

Experiment 1c. HPLC method validation

The HPLC procedure provided by the Sponsor will be validated according to the following criteria:

1. **Specificity**, which will be determined by analyzing a mixture of available standards in the presence and absence of the biological matrix (microsomal incubation mixture).

2. **Linearity**, which will be determined by comparing the radiometric detector response (peak AUC) with the amount of each analyte analyzed in the presence of the biological matrix. The range of these calibration standards to be analyzed will be 1 ng to 10 µg per injection. The results of these experiments will allow construction of a calibration curve, which will be compared with the quality control standards analyzed with each experiment.

3. **Intra-day precision**, which will be determined by repeated analysis of quality control standards on the same day and determination of relative standard deviation of the standard.

4. **Inter-day precision**, which will be determined by repeated analysis of quality control standards on separate days and determination of relative standard deviation of the standard. These data will also provide information on the stability of the analytes in mobile phase.

Experiment 1d. Effect of time, protein and substrate concentration

If Experiment 1b shows metabolism of D_4 by cytochrome P450 and/or FMO, then Experiment 1d will be carried out to determine if metabolite formation is proportional to incubation time and protein concentration. This will help determine whether the metabolites of D_4 are primary metabolites (no lag in formation) or secondary metabolites (lag in formation). Primary metabolites will be selected based on the results of these experiments. This experiment will also ascertain the effects of substrate concentration on the rate of formation of the primary, major, quantifiable metabolite(s), from which the kinetic constants, Km and Vmax, will be determined.

The design of this experiment may be influenced by the results of Experiment 1b, but the overall design will remain essentially the same. D_4 (*e.g.*, 0-10 µM) will be incubated with three concentrations of human liver microsomes (*e.g.*, 0-2.0 mg protein/ml) for a fixed time period (*e.g.*, 0-60 min). Additionally, D_4 (*e.g.*, 0-10 µM) will be incubated with a single concentration of human liver microsomes (*e.g.*, 0.4 mg protein/ml) for multiple time periods (*e.g.*, 0, 5, 10, 15, 20, 30, 45, 60 min). In addition to human liver

microsomes and D_4, the incubation mixtures will contain 50 mM potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM) and β-NADPH (50-250 μM). The remaining procedure will be identical to that described in Experiment 1b. The metabolites of D_4 will be quantified by HPLC as described in Experiment 1a. This experiment will provide the preliminary data necessary to select a range of substrate concentrations and experimental conditions to determine Km and Vmax for the metabolism of D_4 by human liver microsomes. To determine these kinetic constants, Experiment 1c will be repeated with an appropriate range of substrate concentrations under conditions where metabolite formation is proportional to incubation time and protein concentration.

PART 2: Reaction phenotyping: Identification of P450 enzyme(s) in the metabolism of D_4

This and the subsequent experiments will be carried out only if P450 enzymes are responsible for the metabolism of D_4 (determined in Experiment 1b). Reaction phenotyping (*i.e.*, the process of determining which human P450 enzyme or enzymes participate in the metabolism of a chemical) generally involves three types of analysis. These three approaches are:

1. Analysis of the sample-to-sample variation in drug metabolism by a bank of human liver microsomes followed by an analysis of correlations with the sample-to-sample variation in the activity of the major P450 enzymes expressed in human liver microsomes (namely CYP1A2, CYP2A6, CYP2B6 CYP2C9/10, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5 and CYP4A9/11).
2. Analysis of drug metabolism by human liver microsomes in the presence of chemicals or antibodies that inhibit specific P450 enzymes.
3. Analysis of drug metabolism by cDNA-expressed human P450 enzymes.

All procedures will be documented step-by-step and included in the study file.

Experiment 2a. Correlation analysis

D_4 will be incubated with a bank of human liver microsomes to determine inter-individual differences in metabolite formation. The experimental conditions for examining the *in vitro* metabolism of D_4 by this bank of human liver microsomes will be based on the results from Part 1 (especially experiment 1d). Unless indicated otherwise by the Sponsor, the metabolism of D_4 by human liver microsomes will be examined with the lowest possible concentration of D_4 to identify the enzyme(s) responsible for metabolizing D_4 at pharmacologically-relevant concentrations.

This study will be carried out with a bank of human liver microsomes (n = 16) to determine the sample-to-sample variation in the activity of several P450 enzymes (namely CYP1A2, CYP2A6, CYP2B6, CYP2C9/10, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5 and CYP4A9/11). These liver microsomes have

been analyzed according to GLP regulations and the data will be included in the report. Differences in the rates of formation of D_4 metabolites will be compared with the sample-to-sample variation in the following activities:

Enzyme	Enzyme Reaction	SOP #
CYP1A2	7-Ethoxyresorufin *O*-dealkylation	XT12
CYP2A6	Coumarin 7-hydroxylation (measured fluorimetrically)	XT21
CYP2B6	*S*-Mephenytoin *N*-demethylation	XT25 or XT46
CYP2C9	Tolbutamide methyl-hydroxylation	XT24
CYP2C19	S-Mephenytoin 4'-hydroxylation	XT25 or XT46
CYP2D6	Dextromethorphan *O*-demethylation	XT23
CYP2E1	Chlorzoxazone 6-hydroxylation	XT15
CYP3A4/5	Testosterone 6β-hydroxylation	XT16
CYP4A9/11	Lauric acid 12-hydroxylation	XT18 or XT47

Experiment 2b. Chemical and antibody inhibition

Experiment 2b will likely provide information on which human P450 enzyme or enzymes are responsible for metabolizing D_4. The postulated role of a particular P450 enzyme in the metabolism of D_4 can be verified by inhibiting the reaction with chemicals or antibodies known to inhibit that enzyme. Human liver microsomes pooled from several individuals will be used for these studies. Chemical inhibition experiments will be conducted at the lowest possible concentration of D_4. One or more of the following inhibitors will be used to preferentially inhibit certain P450 enzymes:

Enzyme	Reversible inhibitor	Mechanism-based inhibitor
CYP1A2	α-Naphthoflavone (0.1-100 μM)	Furafylline (1-100 μM)
CYP2A6	Nicotine (100-1000 μM)	8-Methoxypsoralen (0.01-10 μM)
CYP2B6	Orphenadrine (100-1000 μM)	
CYP2C9	Sulfaphenazole (1-100 μM)	
CYP2C19	Hexobarbital (10-1000 μM)	
CYP2D6	Quinidine (0.1-100 μM)	
CYP2E1	4-Methylpyrazole (1 -1000 μM)	
CYP3A4/5	Ketoconazole (0.01-1000 μM)	Troleandomycin (10-100 μM)
CYP4A9/11	No known inhibitors are commercially available	

Inhibitory antibodies against CYP1A2, CYP2B6, CYP3A4/5 are available at the Testing Facility, and certain other antibodies are available from commercial sources. These antibodies can be used to investigate the role of individual P450 enzymes in the metabolism of D_4, if such studies are warranted (based on the results of the preceding experiments) and if such studies are deemed necessary by the Sponsor.

Experiment 2c. cDNA-expressed human P450 enzymes

Several human P450 enzymes have been cloned and expressed individually in lymphoblastoid cells. Microsomes from these cells, which contain a single human P450 enzyme, are commercially available from Gentest Corp. (Woburn, MA). If the preceding experiments implicate a particular P450 enzyme in the metabolism of D_4, the ability of that P450 enzyme to metabolize D_4 will be assessed with that cDNA-expressed enzyme. The cDNA-expressed P450 enzymes differ in their catalytic competency, and they are not expressed in lymphoblastoid cells at concentrations that reflect their levels in human liver microsomes. Therefore, they do not establish the *extent* to which a P450 enzyme contributes to the metabolism of a particular drug, only that a particular P450 enzyme *can* metabolize that drug. Also, some of the cDNA-expressed enzymes catalyze certain reactions at rates lower than those catalyzed by human microsomes, whereas some of the P450-enzymes are expressed with additional NADPH-cytochrome P450 reductase. This makes it difficult to interpret the results obtained with cDNA-expressed enzymes. For these reasons, metabolism studies with cDNA-expressed enzymes will be used with the aim of confirming the results obtained from human microsomes.

To assess the ability of one or more cDNA-expressed P450 enzymes to metabolize D_4, experiments similar to those outlined in Experiment 2a will be conducted with the exception that human liver microsomes will be replaced with microsomes from lymphoblastoid cells transfected with cDNA encoding an individual human P450 enzyme.

Source of liver microsomes and antibodies

Liver microsomes from humans are prepared according to SOP XT 03, which is based on the method described by Lu and Levin (1972), and are commercially available through XENOTECH, LLC. These microsomal samples have been extensively characterized with respect to their P450 enzyme activity and the data will be included in the final report. The antibodies against rat CYP1A1, CYP2B1 and CYP3A1 enzymes were raised in male New Zealand White rabbits as described by Thomas *et al.* (1979). The purification and immunoabsorption of these antibodies was carried out by previously described methods (Dutton, 1989; Halvorson, 1990).

Statistical tests and data processing

Data will be processed with a spreadsheet computer program Delta Graph Pro, Version 3.5 (SPSS Inc., Chicago, IL) or Excel (Microsoft, Seattle, WA). For determination of kinetic constants, the data will be analyzed by, Lineweaver Burk and/or Eadie-Hofstee plots (nonlinear regression with appropriate weighting), and the Ki values will be estimated with a computer software GraFit (Version 3.09b or a more recent version, Erithacus Software Limited, London, UK). Correlation analysis, and if necessary, multivariate regression analysis, will be performed with the computer software SigmaStat Statistical Analysis System (Version 2.0 or a more recent version, SPSS Inc., Chicago, IL).

Additional controls

Selected liver microsomal samples will be incubated at half and twice the normal protein concentration and for half and twice the normal incubation period to ascertain whether metabolite formation is directly proportional to enzyme concentration and incubation time. For the P450 enzyme assays, which are initiated by addition of β-NADPH (50-250 μM), concentration of NADPH will be verified spectrophotometrically.

Handling of D_4 and application to the test system:

Upon receipt, D_4 will be stored at ambient (room) temperature either in the original shipping container or in plastic (*i.e.* polypropylene) tubes or vials. During the study, experiments will be performed in either plastic (*i.e.* polypropylene) or Teflon tubes or vials. In the event that D_4 is sealed in crimp-top containers, the septum will have Teflon towards the inside of the container. If a rubber septum is used, aluminum foil will be placed over the vial prior to sealing with the crimp-top to prevent adsorption to the rubber.

D_4 is a liquid with low (50 ppb) solubility in aqueous solutions. Also, D_4 adsorbs to many contact surfaces. Therefore, all concentrations stated in the Protocol will be considered theoretical or target concentrations. In order to determine the true concentration in a given experiment, radiolabeled D_4 will be added to unlabeled D_4 and the specific activity determined. The diluted D_4 will be reconstituted with anhydrous ethanol (McCormick, Weston, MO or equivalent). Ethanol will be stored with molecular sieves (such as, Type 4A grade 514 from Fisher Scientific, St. Louis, MO) to absorb dissolved moisture. Aliquots of ethanol will be drawn with a syringe fitted with a Stainless steel adaptor to filter particulate matter through a glass filter disc. After an aliquot of ethanol is drawn into the syringe, the adaptor will be transferred into plastic (*e.g.*, polypropylene) tubes. Ethanol will then be sampled into a vial containing known amounts of D_4. This solution will serve as the stock of D_4 in anhydrous ethanol, which is stable for at least 4 weeks, in a desiccated container kept at –20°C. The amount of D_4 present in the aqueous solutions will be determined by measuring the amount of radioactivity in the aqueous solutions.

"Typically, for a 1-ml incubation, a mixture containing potassium phosphate buffer (25-100 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM) and microsomal protein (0.01 to 10 mg/ml) will be prepared. Using the stock and substocks (described above), D_4 will be added to the above mixture (final concentration of ethanol will not exceed in 0.5%). The incubations will be started by the addition of β-NADPH (50-250 μM). All incubations will be carried out in plastic (*e.g.*, polypropylene) tubes or vials, all buffer mixtures containing D_4 will be prepared with plastic (*e.g.*, polypropylene) beakers and measuring cylinders. The final concentration of D_4 in the incubation mixture will be checked by sampling the working solutions of D_4 and determining the radioactivity contained therein by liquid scintillation counting. Typically, a 100 to 500 μl aliquot of the mixture will be mixed with 5±0.5 ml of scintillation cocktail and the amount of radioactivity will be estimated."

Procedures and Enzymatic assays:

All procedures will be carried out per the testing facilities Standard Operating Procedures unless stated otherwise in the protocol.

1. The test article will be received, inspected, coded and stored according to SOP XT 27.
2. Buffers will be prepared according to SOP XT 01.
3. HPLC analysis of D_4 and its metabolites will be performed according to the method provided by the Sponsor.

VIII. QUALITY ASSURANCE

The study will be audited by the XenoTech Quality Assurance Unit according to their applicable SOPs.

IX. FINAL REPORT

The final report will include a title page, table of contents, summary of study, quality assurance report, summary of experimental results and overall conclusion, introduction, materials and methods, results, discussion, references, tables of results, figure legend and figures, and various appendices (individual data, approved Protocol, any amendments and deviations).

X. PROCEDURES FOR AMENDING THE PROTOCOL AND REPORTING OF RESULTS

Any change(s) to the approved protocol and reasons for the changes will be documented according to Xenotech SOPs. All unanticipated results, unusual findings and deviations should be reported and documented according to XenoTech's SOP.

XI. RECORDS TO BE MAINTAINED/ARCHIVED

The original versions of all raw and processed data, statistics, documentation, records, protocols, amendments and final reports generated from this study will be archived at the Testing Facility in a locked, fireproof cabinet for 7 years. Due to its instability, raw data printed on thermal paper (HPLC chromatogram and fluorescence measurements) will be photocopied, identified as exact copies of the originals and will be stored with the final report.

XII. REFERENCES

Dutton, D.R. and Parkinson, A.: Reduction of 7-alkoxyresofurins by NADPH-cytochrome P450 reductase and its differential effects on their *O*-dealkylation by rat liver microsomal cytochrome P450. *Arch. Biochem. Biophys.* 268:617-629; 1989.

Halvorson, M., Greenway, D., Eberhart, D., Fitzgerald, K. and Parkinson, A.: Reconstitution of testosterone oxidation by purified rat cytochrome P450p (IIIA1). *Arch. Biochem. Biophys.* 277:166-180; 1990.

Lu, A.Y.H., Levin, W.: Partial purification of cytochrome P-450 and cytochrome P-448 from rat liver microsomes. *Biochem. Biophys. Res. Commun.* 46:1334-1339; 1972.

Pearce, R.E., Coulter, C.J., Madan, A., Sanzgiri, U., Draper, A.J., Bullock, P.L., Cook, D.C., Burton, L.A., Latham, J.L., Nevins, C.J., and Parkinson, A.: Effects of freezing, thawing and storing human liver microsomes on cytochrome P450 activity. *Arch. Biochem. Biophys.* 331:145-169; 1996.

Thomas, P.E., Korzenioski, D.E., Ryan, D.E. and Levin, W.: Preparation of monospecific antibodies against two forms of rat liver cytochrome P450 and quantitation of these antigens in microsomes. *Arch. Biochem. Biophys.* 192:524-532; 1979.

XIII. **PROTOCOL APPROVAL**

Etsuko Usuki 6/29/99

Etsuko Usuki, Ph.D.
Study Director
XenoTech LLC
Kansas City, KS

Date

6/28/99

Ajay Madan, Ph.D.
Study Manager
XenoTech LLC
Kansas City, KS

Date

Kathy P Plotzke 6-24-99

Kathy Plotzke, Ph.D.
Sponsor Representative/Study Monitor
Toxicology Research Manager
Dow Corning Corporation
Midland, MI

Date

Protocol Amendment

Amendment:	1
Date Issued:	August 3, 1999
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents changes to two sections in the protocol as described below.

Amendment:
1. Section II, Page 2, Personnel involved in the study: Thomas L.F. White, B.S. (Associate Toxicologist) is added to the section as the Sponsor/Study Coordinator.
2. Section III, Page 3, Proposed Test Schedule: Replace "To be determined" in 4 places of this section with the dates as following.

Proposed experimental start date:	July 12, 1999
Proposed experimental termination date:	February 1, 2000
Target date for submission of draft unsigned report:	March 1, 2000
Target date for submission of final report:	Within three weeks of receiving the Sponsor's comments on the draft report

Reasons for Amendment:
1. Sponsor's request (letter dated June 30, 1999)
2. Schedule was not determined when the protocol was signed. Schedule was submitted and approved by the Sponsor (letter dated July 12, 1999).

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

8/3/99
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

8/4/99
Date

Protocol Amendment

Amendment:	2
Date Issued:	October 14, 1999
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents changes in "Experiment 1c. HPLC method validation" and the use of quality control standards.

Amendment:
1. Linearity of the detector response: The range of the calibration standards to be analyzed will be 40 pmol to 400 pmol per injection (0.4 µM to 4.0 µM in incubation) instead of 1 ng to 10 µg per injection.
2. Inter-day and Intra-day precision will be determined by repeated analysis of the zero-time incubation samples at three concentrations. However, we will not have a pool of quality control samples to be analyzed for each experiment in the study.

Reasons for Amendment:
1. Since the target concentration of the test article in this protocol is 3 µM, it is not necessary for the calibration curve to exceed 3 µM. The Testing Facility decided to cover the range up to 4 µM.
2. The protocol states that quality control samples will be generated and compared to the standard curve for each experiment. However, due to the nature of D_4, quality controls samples will not be produced; instead, the activity of each substrate mixture will be determined by liquid scintillation counting for all experiments prior to sample incubations.

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

10/20/99
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

18 Oct 99
Date

Protocol Amendment

Amendment:	3
Date Issued:	December 20, 1999
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents a change in the order of experiments to be carried out as described in the protocol (pages 7-9).

Amendment:
Experiment 2c "cDNA-expressed human P450 enzymes" will be performed prior to "Km and Vmax determination" (part of Experiment 1d), Experiment 2a "Correlation analysis" and Experiment 2b "Chemical and antibody inhibition".

Reasons for Amendment:
The amount of metabolite formation observed in Experiment 1d "Effect of time, protein and substrate concentration" was not proportional to incubation time and protein concentration. Although the metabolite formation was β-NADPH dependent and was not observed in zero-time incubations, in order to reassess whether or not the metabolite formation was catalyzed by cytochrome P450 enzymes, an experiment with cDNA-expressed P450s will be performed prior to other experiments (*i.e.*, Km and Vmax determination, correlation analysis, chemical and antibody inhibition), that were originally designed to be carried out prior to the cDNA experiment.

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

1/3/00
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

12/23/99
Date

Protocol Amendment

Amendment:	4
Date Issued:	January 27, 2000
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents changes in Section VI, entitled "TEST ARTICLE SPECIFICATION" (Page 3).

Amendment:
1. Replace "To be determined" for the lot number with "Lot No. 990316".
2. Replace "To be documented in the study file and the final report" for the specific activity with "20.62 mCi/mmol".
3. Replace "To be documented in the study file and the final report" for the purity with "99.67%".
4. Replace "To be determined" for the concentrations of substocks with "9.7 mM".
5. Although Section VI lists D_4-unlabeld as a test article, it has never been (and will not be) used in the study.

Reasons for Amendment:
1. Information became available upon sample receipt.
2. Information became available upon sample receipt.
3. Information became available upon sample receipt.
4. The concentration of the substock solution was determined by XenoTech based on the result of scintillation counting of aliquots of the substock solution and the specific activity.
5. It was determined after the protocol was signed that only radiolabeled D_4 to be used in the study.

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

2/4/00
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

01 Feb 00
Date

Protocol Amendment

Amendment:	5
Date Issued:	February 22, 2000
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents an additional experiment to assess the possibility of D_4 or a metabolite binding to protein.

Amendment:
The possibility of D_4 or a metabolite binding to protein will be assessed as following:

1. Substrate buffer-mix containing human liver microsomes, potassium phosphate buffer, EDTA, $MgCl_2$, water will be prepared.
2. One hundred microliter of NADPH-generating system will be added to 700 µl of the substrate buffer-mix. Immediately after these solutions are mixed, radioactivity of an aliquot of the mixture will be measured by scintillation counting (this sample contains no THF).
3. Carry out standard incubations and stop the reaction with THF at designated incubation time (*e.g.*, 0, 20, 40 and 60 min). Remove 100-µl aliquot of the solution and count the radioactivity.
4. Centrifuge the samples to separate microsomal pellet and supernatant fraction.
5. Count the radioactivity in the supernatant fraction. Solubilize the pelleted protein and count the radioactivity to see if there is radioactivity bound to the protein.

Reasons for Amendment:
Previous data (Assessment of time and protein proportionality) showed discrepancy in the amount of D_4 (substrate) lost during the course of incubation and the amount of metabolite formed. In order to evaluate the ^{14}C mass balance, this experiment was designed.

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

3/10/00
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

3-7-00
Date

Protocol Amendment

Amendment:	6
Date Issued:	March 16, 2000
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents an additional experiment to assess effect of incubation time and protein concentration on the metabolism of D_4 by Phenobarbital-treated rat liver microsomes. Phenobarbital-treated rat liver microsomes (0.25, 0.5 and 1.0 mg protein/mL) will be incubated at 37±1°C in incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4±0.1), $MgCl_2$ (3 mM), EDTA (1 mM) and D_4 (3 µM) with β-NADPH for 30 min. In addition, D_4 (3 µM) will be incubated with Phenobarbital-treated rat liver microsomes (0.5 mg protein/mL) for 15, 30 and 60 min. Zero-time, zero-protein and zero-substrate incubations will serve as blanks. Furthermore, D_4 (3 µM) will be incubated with Phenobarbital-treated rat liver microsomes (0.5 mg protein/mL) in the absence of β-NADPH for 0 and 30 min. Precipitated protein will be removed by centrifugation (400-2,500 *g* for 5-15 min at 5-15°C). An aliquot (up to 500 µl) of the supernatant fraction will be analyzed by HPLC.

Amendment:
In order to assess the effect of incubation time and protein concentration on the metabolism of D_4 by rat liver microsomes, D_4 (3 µM) will be incubated with Phenobarbital-treated rat liver microsomes.

Reasons for Amendment:
Previous data (assessment of time and protein proportionality using human liver microsomes) showed discrepancy in the amount of D_4 (substrate) lost during the course of incubation and the amount of metabolite formed. It is known that Phenobarbital-treated rat liver microsomes metabolize D_4 extensively (Sponsor's internal report), however, the effect of time and protein has not been examined. Thus, this experiment was designed to assess the effect of time and protein using Phenobarbital-treated rat liver microsomes.

APPROVAL:

Etsuko Usuki

Etsuko Usuki, Ph.D.
Study Director

3/21/00
Date

Kathy P Plotzke

Kathy Plotzke, Ph.D.
Sponsor's Monitor

3/17/00
Date

Protocol Amendment

Amendment:	7
Date Issued:	April 17, 2000
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Principal Scientist:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Amendment:
This amendment documents an additional experiment to assess the difference in the metabolism of D_4 between Phenobarbital-treated rat liver microsomes and control (saline-treated) rat liver microsomes. Phenobarbital-treated rat liver microsomes (up to 0.5 mg/mL) and control rat liver microsomes (up to 0.5 mg/mL) will be incubated at 37±1°C in incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4±0.1), $MgCl_2$ (3 mM), EDTA (1 mM) and D_4 (up to 5 µM) with ß-NADPH for up to 30 minutes. Precipitated protein will be removed by centrifugation (400-2,500 *g* for 5-15 min at 5-15°C). An aliquot (up to 500 µL) of the supernatant fraction will be analyzed by HPLC.

Reason for Amendment:
Previous data (assessment of time and protein course using Phenobarbital induced Rat microsomes, Amendment-6) showed complete metabolism of D_4 (3 µM) at protein concentrations as low as 0.25 mg/mL for an incubation time of 15 minutes. This experiment will assess the difference in the metabolism of D_4 (up to 5 µM) on both Phenobarbital-treated rat liver microsomes and control rat liver microsomes.

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

4/21/00
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

18 April 2000
Date

Protocol Amendment

Amendment:	8
Date Issued:	July 24, 2000
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Amendment:
This amendment documents an additional experiment to assess effect of incubation time and amount of P450 enzyme on the metabolism of D_4 by human recombinant CYP2B6 and CYP3A4. Recombinant CYP2B6 and CYP3A4 (25, 50, 75 and 100 pmol/incubation) will be incubated at 37±1°C in incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4±0.1), $MgCl_2$ (3 mM), EDTA (1 mM) and D_4 (3 µM) with β-NADPH for 60 min. In addition, D_4 (3 µM) will be incubated with recombinant CYP2B6 and CYP3A4 (100 pmol/incubation) for 30, 60, 90 and 120 min. Zero-time and zero-protein incubations will serve as blanks. Precipitated protein will be removed by centrifugation (400-2,500 *g* for 5-15 min at 5-15°C). An aliquot (up to 500 µL) of the supernatant fraction will be analyzed by HPLC.

Reasons for Amendment:
Previous data (assessment of time and protein linearity using human liver microsomes) showed no linear relationship between the metabolite formation and incubation time/protein concentration. However, the cDNA experiment showed that CYP2B6 and CYP3A4 catalyzed this metabolite formation. Therefore, it was determined that the linearity should be reassessed using these recombinant P450s.

APPROVAL:

Etsuko Usuki
Etsuko Usuki, Ph.D.
Study Director

7/24/00
Date

Kathy P. Plotzke
Kathy Plotzke, Ph.D.
Sponsor's Monitor

7/28/00
Date

Protocol Amendment

Amendment:	9
Date Issued:	August 29, 2000
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Amendment:
This amendment documents an additional experiment to generate incubation samples of D_4 with a pool of human liver microsomes. Human liver microsomes (0.5 mg/mL) will be incubated in crimp-top vials at 37±1°C in incubation mixtures (final incubation volume of 800 µL) containing potassium phosphate buffer (50 mM, pH 7.4±0.1), $MgCl_2$ (3 mM), EDTA (1 mM) and D_4 (3 µM) with β-NADPH for 60 min. Zero-time and no-β-NADPH incubations will serve as blanks. Protein will be precipitated by centrifugation (400-2,500 *g* for 5-15 min at 5-15°C). These samples will be shipped to the sponsor for possible future analysis. The samples will be shipped on dry ice by overnight courier. The sponsor will document any analysis and prepare a memo report to include in the study records.

APPROVAL:

Etsuko Usuki
Etsuko Usuki, Ph.D.
Study Director

9/5/00
Date

Kathy Plotzke
Kathy Plotzke, Ph.D.
Sponsor's Monitor

9-1-00
Date

Protocol Amendment

Amendment:	10
Date Issued:	March 12, 2001
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents a change in the personnel involved in the study as described below.

Amendment:
The Study Coordinator was changed from Thomas L.F. White, B.S. to Linda Meeker, B.S.

Reasons for Amendment:
Sponsor's request (telephone conversation and e-mail dated March 9, 2001)

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

3/20/01
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

3-14-01
Date

Protocol Amendment

Amendment:	11
Date Issued:	June 21, 2001
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents changes in the experimental design in the following section.

- Experiment 1d. Effect of time, protein and substrate concentration
- Experiment 2a. Correlation analysis
- Experiment 2b. Chemical and antibody inhibition

Amendment:
- Km, Vmax determination (part of Experiment 1d) will not be performed.
- Correlation analysis will not be performed.
- Chemical inhibition (part of Experiment 2b) will not be performed.

Reasons for Amendment:
Based on the collected data, it was decided (on 4/27/01 at the meeting with the Sponsor) that no further experiment would benefit the study. Since D_4 appeared to be metabolically stable and the metabolite formation did not show proportionality with respect to incubation time and protein concentration, these three experiments listed above would not provide any additional information to that the Testing Facility has already documented.

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

6/25/01
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

6-22-01
Date

Protocol Amendment

Amendment:	12
Date Issued:	June 21, 2001
Protocol No.:	XT052398 (Dow Corning Study No. 8956)
Protocol title:	*In vitro* metabolism of octamethylcyclotetrasiloxane (D_4) by human liver microsomes
Testing Facility:	XenoTech L.L.C., Kansas City, KS
Study Director:	Etsuko Usuki, Ph.D.
Study Manager:	Ajay Madan, Ph.D.
Sponsor:	Dow Corning Corporation, Midland, MI
Sponsor's Monitor:	Kathy Plotzke, Ph.D.

Sections Amended:
This amendment documents a change to Section III of the protocol as described below.

Amendment:
Section III, Page 3, Proposed Test Schedule: Replace the schedule (specifically "Target date for submission of draft unsigned report") described in Amendment-1 (issued 8/3/99) with the date as following.

Proposed experimental start date:	July 12, 1999
Proposed experimental termination date:	February 1, 2000
Target date for submission of draft unsigned report:	July 13, 2001
Target date for submission of final report:	Within three weeks of receiving the Sponsor's comments on the draft report

Reasons for Amendment:
The last experiment was performed at the Testing Facility on September 7, 2000 (sample preparation for HPLC analysis by the Sponsor, documented in Amendment-9). Previous target date for submission of draft unsigned report was March 1, 2000 and the target date was necessary to be revised.

APPROVAL:

Etsuko Usuki, Ph.D.
Study Director

6/25/01
Date

Kathy Plotzke, Ph.D.
Sponsor's Monitor

6-22-01
Date

Appendix 6: Tabular summary of data

Time and Protein-1 (Human liver microsomes)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/inc.)	Rounded Rate of metabolite formation (pmol/mg/min)
3 µM No Protein	ND	60	0	ND
3 µM No ß-NADPH	ND	60	0.8	ND
3 µM 0.5 x Protein	187	60	0.4	7.80
3 µM 1 x Protein	248	60	0.8	5.18
3 µM 2 x Protein	214	60	1.6	2.23
3 µM D4	ND	0	0.8	ND
3 µM D4	232	15	0.8	19.4
3 µM D4	251	30	0.8	10.5
3 µM D4	223	45	0.8	6.19
3 µM D4	233	60	0.8	4.86
3 µM D4	240	120	0.8	2.50
5 µM D4	ND	0	0.8	ND
5 µM D4	326	30	0.8	13.6
5 µM D4	290	60	0.8	6.03
10 µM D4	162	0	0.8	ND
10 µM D4	505	30	0.8	21.1
10 µM D4	489	60	0.8	10.2
3 µM, 2 x Protein	187	120	1.6	0.975

Values are mean of duplicate determinations
ND: Not determined
Rate of metabolite formation = Metabolite formed per incubation (pmol)/mg protein per incubation/min of incubation time
Rounded rate of metabolite formation: Rounded to three significant figures

Experiment was performed on 11/18/99

Effect of time and protein on M1 formation (Species comparison-2)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/inc.)	Rounded Rate (pmol/mg/min)
0 x Protein	ND	30	0	ND
0 x T Human	ND	0	0.4	ND
0.5 x T Human	ND	15	0.4	ND
1 x T Human	ND	30	0.4	ND
2 x T Human	ND	60	0.4	ND
0.5 x P Human	ND	30	0.2	ND
1 x P Human	ND	30	0.4	ND
2 x P Human	ND	30	0.8	ND
No NADPH Human	ND	30	0.4	ND
0 x T ST Rat	ND	0	0.2	ND
0.5 x T ST Rat	ND	5	0.2	ND
1 x T ST Rat	ND	10	0.2	ND
2 x T ST Rat	ND	20	0.2	ND
0.5 x P ST Rat	ND	10	0.1	ND
1 x P ST Rat	ND	10	0.2	ND
2 x P ST Rat	ND	10	0.4	ND
No NADPH ST Rat	ND*	10	0.2	ND
0 x T PB Rat	ND	0	0.2	ND
0.5 x T PB Rat	ND	5	0.2	ND
1 x T PB Rat	489.91	10	0.2	245
2 x T PB Rat	788.67	20	0.2	197
0.5 x P PB Rat	210.26	10	0.1	210
1 x P PB Rat	582.92	10	0.2	291
2 x P PB Rat	611.95	10	0.4	153
No NADPH PB Rat	ND	10	0.2	ND

Rounded rate: Metabolite formed per incubation (pmol)/mg protein per inc/incubation time (min), rounded to three significant figures.
ND: under metabolite formation column = not detected, under rate column = not determined
ND*: Not determined due to misinjection
T: Time, P: Protein
ST Rat: Saline-treated rats
PB Rat: Phenobarbital-treated rats

Experiment was performed on 6/22/00

Effect of time and protein on M2 formation (Species comparison-2)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/inc.)	Rounded Rate (pmol/mg/min)
0 x Protein	ND	30	0	ND
0 x T Human	ND	0	0.4	ND
0.5 x T Human	ND	15	0.4	ND
1 x T Human	ND	30	0.4	ND
2 x T Human	ND	60	0.4	ND
0.5 x P Human	ND	30	0.2	ND
1 x P Human	ND	30	0.4	ND
2 x P Human	ND	30	0.8	ND
No NADPH Human	ND	30	0.4	ND
0 x T ST Rat	ND	0	0.2	ND
0.5 x T ST Rat	ND	5	0.2	ND
1 x T ST Rat	ND	10	0.2	ND
2 x T ST Rat	ND	20	0.2	ND
0.5 x P ST Rat	ND	10	0.1	ND
1 x P ST Rat	ND	10	0.2	ND
2 x P ST Rat	ND	10	0.4	ND
No NADPH ST Rat	ND*	10	0.2	ND
0 x T PB Rat	ND	0	0.2	ND
0.5 x T PB Rat	ND	5	0.2	ND
1 x T PB Rat	137.66	10	0.2	68.8
2 x T PB Rat	118.36	20	0.2	29.6
0.5 x P PB Rat	ND	10	0.1	ND
1 x P PB Rat	82.79	10	0.2	41.4
2 x P PB Rat	143.48	10	0.4	35.9
No NADPH PB Rat	ND	10	0.2	ND

Rounded rate: Metabolite formed per incubation (pmol)/mg protein per Inc/incubation time (min), rounded to three significant figures.
ND: under metabolite formation column = not detected, under rate column = not determined
ND*: Not determined due to misinjection
T: Time, P: Protein
ST Rat: Saline-treated rats
PB Rat: Phenobarbital-treated rats

Experiment was performed on 6/22/00

Effect of time and protein on M3 formation (Species comparison-2)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/Inc.)	Rounded Rate (pmol/mg/min)
0 x Protein	ND	30	0	ND
0 x T Human	ND	0	0.4	ND
0.5 x T Human	ND	15	0.4	ND
1 x T Human	ND	30	0.4	ND
2 x T Human	ND	60	0.4	ND
0.5 x P Human	ND	30	0.2	ND
1 x P Human	ND	30	0.4	ND
2 x P Human	ND	30	0.8	ND
No NADPH Human	ND	30	0.4	ND
0 x T ST Rat	ND	0	0.2	ND
0.5 x T ST Rat	ND	5	0.2	ND
1 x T ST Rat	ND	10	0.2	ND
2 x T ST Rat	ND	20	0.2	ND
0.5 x P ST Rat	ND	10	0.1	ND
1 x P ST Rat	ND	10	0.2	ND
2 x P ST Rat	ND	10	0.4	ND
No NADPH ST Rat	ND*	10	0.2	ND
0 x T PB Rat	ND	0	0.2	ND
0.5 x T PB Rat	123.35	5	0.2	123
1 x T PB Rat	272.80	10	0.2	136
2 x T PB Rat	433.06	20	0.2	108
0.5 x P PB Rat	201.01	10	0.1	201
1 x P PB Rat	290.74	10	0.2	145
2 x P PB Rat	267.90	10	0.4	67.0
No NADPH PB Rat	ND	10	0.2	ND

Rounded rate: Metabolite formed per incubation (pmol)/mg protein per inc/incubation time (min), rounded to three significant figures.
ND: under metabolite formation column = not detected, under rate column = not determined
ND*: Not determined due to misinjection
T: Time, P: Protein
ST Rat: Saline-treated rats
PB Rat: Phenobarbital-treated rats

Experiment was performed on 6/22/00

Effect of time and protein on M4 formation (Species comparison-2)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/inc.)	Rounded Rate (pmol/mg/min)
0 x Protein	ND	30	0	ND
0 x T Human	ND	0	0.4	ND
0.5 x T Human	ND	15	0.4	ND
1 x T Human	ND	30	0.4	ND
2 x T Human	ND	60	0.4	ND
0.5 x P Human	ND	30	0.2	ND
1 x P Human	ND	30	0.4	ND
2 x P Human	ND	30	0.8	ND
No NADPH Human	ND	30	0.4	ND
0 x T ST Rat	ND	0	0.2	ND
0.5 x T ST Rat	ND	5	0.2	ND
1 x T ST Rat	ND	10	0.2	ND
2 x T ST Rat	ND	20	0.2	ND
0.5 x P ST Rat	ND	10	0.1	ND
1 x P ST Rat	ND	10	0.2	ND
2 x P ST Rat	ND	10	0.4	ND
No NADPH ST Rat	ND*	10	0.2	ND
0 x T PB Rat	ND	0	0.2	ND
0.5 x T PB Rat	91.37	5	0.2	91.4
1 x T PB Rat	215.92	10	0.2	108
2 x T PB Rat	280.53	20	0.2	70.1
0.5 x P PB Rat	90.51	10	0.1	90.5
1 x P PB Rat	187.83	10	0.2	93.9
2 x P PB Rat	164.06	10	0.4	41.0
No NADPH PB Rat	ND	10	0.2	ND

Rounded rate: Metabolite formed per incubation (pmol)/mg protein per inc/incubation time (min), rounded to three significant figures.
ND: under metabolite formation column = not detected, under rate column = not determined
ND*: Not determined due to misinjection
T: Time, P: Protein
ST Rat: Saline-treated rats
PB Rat: Phenobarbital-treated rats

Experiment was performed on 6/22/00

Effect of time and protein on M5 formation (Species comparison-2)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/inc.)	Rounded Rate (pmol/mg/min)
0 x Protein	ND	30	0	ND
0 x T Human	ND	0	0.4	ND
0.5 x T Human	ND	15	0.4	ND
1 x T Human	ND	30	0.4	ND
2 x T Human	ND	60	0.4	ND
0.5 x P Human	ND	30	0.2	ND
1 x P Human	ND	30	0.4	ND
2 x P Human	99.94	30	0.8	4.16
No NADPH Human	ND	30	0.4	ND
0 x T ST Rat	ND	0	0.2	ND
0.5 x T ST Rat	ND	5	0.2	ND
1 x T ST Rat	ND	10	0.2	ND
2 x T ST Rat	86.45	20	0.2	21.6
0.5 x P ST Rat	ND	10	0.1	ND
1 x P ST Rat	ND	10	0.2	ND
2 x P ST Rat	ND	10	0.4	ND
No NADPH ST Rat	ND*	10	0.2	ND
0 x T PB Rat	ND	0	0.2	ND
0.5 x T PB Rat	95.81	5	0.2	95.8
1 x T PB Rat	131.57	10	0.2	65.8
2 x T PB Rat	ND	20	0.2	ND
0.5 x P PB Rat	ND	10	0.1	ND
1 x P PB Rat	110.54	10	0.2	55.3
2 x P PB Rat	145.27	10	0.4	36.3
No NADPH PB Rat	ND	10	0.2	ND

Rounded rate: Metabolite formed per incubation (pmol)/mg protein per inc/incubation time (min), rounded to three significant figures.
ND: under metabolite formation column = not detected, under rate column = not determined
ND*: Not determined due to misinjection
T: Time, P: Protein
ST Rat: Saline-treated rats
PB Rat: Phenobarbital-treated rats

Experiment was performed on 6/22/00

Effect of time and protein on M6 formation (Species comparison-2)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/inc.)	Rounded Rate (pmol/mg/min)
0 x Protein	ND	30	0	ND
0 x T Human	ND	0	0.4	ND
0.5 x T Human	ND	15	0.4	ND
1 x T Human	ND	30	0.4	ND
2 x T Human	ND	60	0.4	ND
0.5 x P Human	ND	30	0.2	ND
1 x P Human	ND	30	0.4	ND
2 x P Human	ND	30	0.8	ND
No NADPH Human	ND	30	0.4	ND
0 x T ST Rat	ND	0	0.2	ND
0.5 x T ST Rat	ND	5	0.2	ND
1 x T ST Rat	ND	10	0.2	ND
2 x T ST Rat	ND	20	0.2	ND
0.5 x P ST Rat	ND	10	0.1	ND
1 x P ST Rat	ND	10	0.2	ND
2 x P ST Rat	ND	10	0.4	ND
No NADPH ST Rat	ND*	10	0.2	ND
0 x T PB Rat	ND	0	0.2	ND
0.5 x T PB Rat	ND	5	0.2	ND
1 x T PB Rat	90.30	10	0.2	45.2
2 x T PB Rat	128.79	20	0.2	32.2
0.5 x P PB Rat	ND	10	0.1	ND
1 x P PB Rat	113.94	10	0.2	57.0
2 x P PB Rat	261.37	10	0.4	65.3
No NADPH PB Rat	ND	10	0.2	ND

Rounded rate: Metabolite formed per incubation (pmol)/mg protein per inc/incubation time (min), rounded to three significant figures.
ND: under metabolite formation column = not detected, under rate column = not determined
ND*: Not determined due to misinjection
T: Time, P: Protein
ST Rat: Saline-treated rats
PB Rat: Phenobarbital-treated rats

Experiment was performed on 6/22/00

Effect of time and protein on M7 formation (Species comparison-2)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/inc.)	Rounded Rate (pmol/mg/min)
0 x Protein	ND	30	0	ND
0 x T Human	ND	0	0.4	ND
0.5 x T Human	ND	15	0.4	ND
1 x T Human	ND	30	0.4	ND
2 x T Human	ND	60	0.4	ND
0.5 x P Human	ND	30	0.2	ND
1 x P Human	ND	30	0.4	ND
2 x P Human	ND	30	0.8	ND
No NADPH Human	ND	30	0.4	ND
0 x T ST Rat	ND	0	0.2	ND
0.5 x T ST Rat	ND	5	0.2	ND
1 x T ST Rat	ND	10	0.2	ND
2 x T ST Rat	ND	20	0.2	ND
0.5 x P ST Rat	ND	10	0.1	ND
1 x P ST Rat	ND	10	0.2	ND
2 x P ST Rat	ND	10	0.4	ND
No NADPH ST Rat*	ND	10	0.2	ND
0 x T PB Rat	ND	0	0.2	ND
0.5 x T PB Rat	ND	5	0.2	ND
1 x T PB Rat	204.93	10	0.2	102
2 x T PB Rat	466.76	20	0.2	117
0.5 x P PB Rat	ND	10	0.1	ND
1 x P PB Rat	302.14	10	0.2	151
2 x P PB Rat	534.34	10	0.4	134
No NADPH PB Rat	ND	10	0.2	ND

Rounded rate: Metabolite formed per incubation (pmol)/mg protein per inc/incubation time (min), rounded to three significant figures.
ND: under metabolite formation column = not detected, under rate column = not determined
ND*: Not determined due to misinjection
T: Time, P: Protein
ST Rat: Saline-treated rats
PB Rat: Phenobarbital-treated rats

Experiment was performed on 6/22/00

Effect of time and protein on M8 formation (Species comparison-2)

Sample	Metabolite formed per incubation (pmol)	Incubation time (min)	Protein (mg/inc.)	Rounded Rate (pmol/mg/min)
0 x Protein	ND	30	0	ND
0 x T Human	ND	0	0.4	ND
0.5 x T Human	217.64	15	0.4	36.3
1 x T Human	234.14	30	0.4	19.5
2 x T Human	212.35	60	0.4	8.85
0.5 x P Human	139.63	30	0.2	23.3
1 x P Human	253.81	30	0.4	21.2
2 x P Human	213.85	30	0.8	8.91
No NADPH Human	ND	30	0.4	ND
0 x T ST Rat	ND	0	0.2	ND
0.5 x T ST Rat	140.82	5	0.2	141
1 x T ST Rat	172.57	10	0.2	86.3
2 x T ST Rat	172.30	20	0.2	43.1
0.5 x P ST Rat	109.85	10	0.1	110
1 x P ST Rat	180.15	10	0.2	90.1
2 x P ST Rat	266.21	10	0.4	66.6
No NADPH ST Rat	ND*	10	0.2	ND
0 x T PB Rat	ND	0	0.2	ND
0.5 x T PB Rat	647.94	5	0.2	648
1 x T PB Rat	570.27	10	0.2	285
2 x T PB Rat	205.02	20	0.2	51.3
0.5 x P PB Rat	356.45	10	0.1	356
1 x P PB Rat	589.73	10	0.2	295
2 x P PB Rat	216.37	10	0.4	54.1
No NADPH PB Rat	ND	10	0.2	ND

Rounded rate: Metabolite formed per incubation (pmol)/mg protein per inc/incubation time (min), rounded to three significant figures.
ND: under metabolite formation column = not detected, under rate column = not determined
ND*: Not determined due to misinjection
T: Time, P: Protein
ST Rat: Saline-treated rats
PB Rat: Phenobarbital-treated rats

Experiment was performed on 6/22/00

Appendix 7: Memorandum; HPLC method optimization results (Amendment 9)

XT052398

Memorandum

To: Etsuko Usuki, Senior Scientist, Principle Scientist
XenoTech, LLC

CC: Study 8956 (XT052398) file, Kathleen Plotzke, Toxicology Manager, Sponsors Representative, Dow Corning Corp.

From: Thomas L. F. White, Associate Toxicologist, Study Coordinator, Dow Corning Corporation

Date: 10/29/2000

Re: HPLC method optimization results

Dow Corning's Toxicology Laboratory received from XenoTech LLC, samples derived from microsomal incubations (^{14}C-D_4 and Human Liver Microsomes). A 75 µl aliquot from each sample was analyzed by HPLC with radiometric detection in an attempt to optimize analysis conditions. DCC personnel evaluated the resulting chromatographic profiles. The work was conduced as non-regulated method development.

Only parent compound was present in chromatograms of samples representing 3µM D4 incubated with 0.5mg/ml HLM (human liver microsomes) 0min and 3µM D4 incubated for 60min. with 0.5mg/ml HLM without β-NADPH. Parent compound and a single metabolite were observed in chromatograms from samples representing 3µM D4 incubated with 0.5mg/ml HLM incubated for 60min. in the presence of β-NADPH.

Based on review of the chromatograms no further optimization is suggested at this time. The profile achieved by the DCC analysis is consistent with that achieved by XenoTech. No other parameters of the samples were evaluated.

All raw data including but not limited to chromatograms, methods and sample information will be archived in the study file at Dow Corning Corporation 2200 W Salzburg Rd. Midland, MI 48606-0994.

Respectfully Submitted,

Thomas L. F. White

Thomas L. F. White (Tim) B.S.
Associate Toxicologist
Biomolecular Toxicology
Dow Corning Corporation
Office 517.496.1762

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